Cross-bridging the gap between energetics, Ca2+, sarcomere length and diastolic dysfunction

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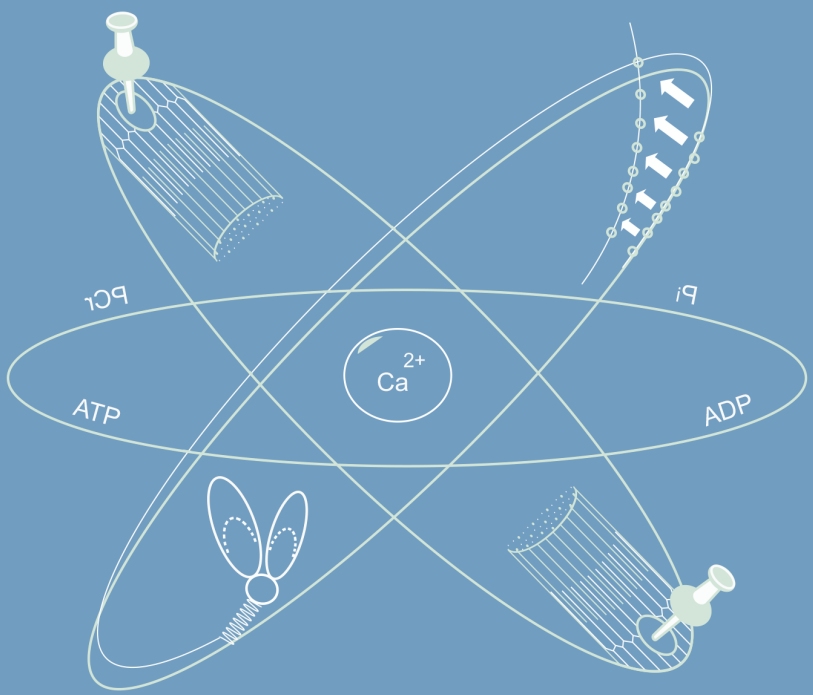

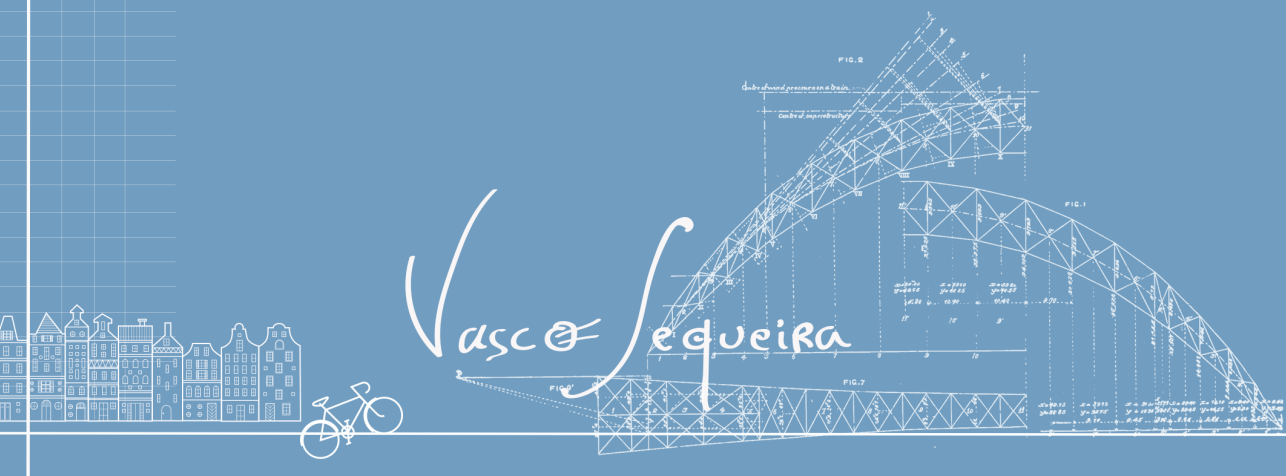

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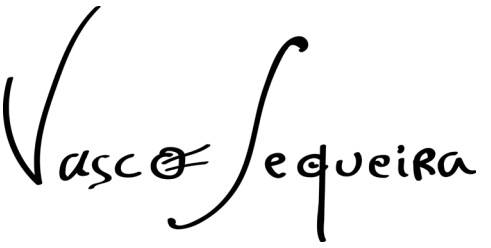

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Cross-bridging the gap between energetics, Ca^{2+}, sarcomere length and diastolic dysfunction

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geboren te Porto, Portugal

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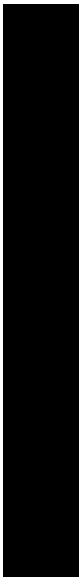

REGULATION OF LENGTH-DEPENDENT ACTIVATION, THE CELLULAR BASIS OF THE STARLING LAW OF THE HEART

One that initiates a PhD research project, for which I include my teenage self and many of my friends, wrongly assumes that the current knowledge in all fields of Science is only a few decades old and that all fundamental knowledge about Science is well known to man. This leads to the unfortunate situation of forgetting the persons that paved the ways in Science, and additionally may discourage a newcomer to initiate a research project. However, and importantly, one should learn from history that our current understanding of Nature is by no means only decades old, has not stopped, and that it is the convergence of intellectual knowledge gathered over hundreds and hundreds years of research. I find the words of William Thomson at the end of the 19th century the best way to illustrate the above. In 1900 the British physicist and engineer William Thomson better known as Lord Kelvin, when addressing the British Association for the Advancement of Science, remarked that in physics there were no more fundamental discoveries to be made.

> *There is nothing new to be discovered in physics now. All that remains is more and more precise measurement.*

Kelvin made important contributions to the analysis of electricity and the formulation of the 1st and 2nd laws of thermodynamics and is best known for determining the precise value of the lowest temperature existing in the universe (absolute zero). This value approximates -273°C; Units of Kelvin, a measure of temperature, named in his honor. Lord Kelvin's quote always comes to mind, because I believe that nowadays we share the same erroneous belief as those that lived hundreds of years ago. Lord Kelvin's remark may have been triggered by the discoveries of several fundamental Laws of Nature by Nicolaus Copernicus, Galileu Galilei, Isaac Newton, Michael Faraday, James Clerk Maxwell to name a few. However, what history teaches us over and over again is that his remark could not be more wrong. For instance, just 5 years after Kelvin spoke those words, a young German graduate physicist living in Bern, Switzerland, at the age of 26, published 4 papers, which are considered some of the most important contributions to Physics and Science of all time. His name was Albert Einstein. The year of 1905 became known as "*Annus mirabilis*" from the Latin "miraculous year" of Einstein, and Special/General relativity would change the Newtonian view of the universe forever. I do not intend to end and conclude with that we should all be Albert Einstein, but instead that we should be open to the idea that there is still more exciting and fundamental knowledge to be discovered, that complexity should not discourage us, and that there is a lot to be gained from pursuing ones dreams.

I hope you will enjoy reading the fundamental studies I performed during my PhD, for which I also desire to understand Nature's Laws, secrets and the remarkable complexity attached to it. I hope the generalizations of my findings provoke the exchange of constructive ideas among researchers everywhere.

1

INTRODUCTION WITH THESIS OUTLINE

"It's not that I'm smart, it's just that I stay with problems longer."

Albert Einstein

HEART FAILURE AND DIASTOLIC DYSFUNCTION

In Europe over 15 million people are diagnosed with heart failure (HF) and this number continues to rapidly increase, representing a major cause of hospitalization and death.[1] HF is a clinical syndrome defined as the inability of the heart to sufficiently supply blood to organs and tissues.[2] About half of the HF patient population presents with contractile dysfunction and ventricular dilation (HF with reduced ejection fraction). The other half of the HF patient population has a heart which is unable to properly relax and distend.[3] These patients show near normal contractile function and often hypertrophy of the heart, and are characterized by abnormal diastolic function (HF with preserved ejection fraction, HFpEF).[3]

Diastolic dysfunction does not solely affect non-familial cardiac disorders as it is a common defect in patients with cardiomyopathies. Hypertrophic cardiomyopathy (HCM) is the most common inherited form of cardiomyopathy, frequently caused by gene mutations encoding sarcomere proteins and characterized by asymmetric hypertrophy.[4] HCM-causing mutations that affect genes encoding the thick- and thin-filament proteins account for ~98% of all HCM mutations reported thus far in humans.[5] Importantly, both animal[6,7] and clinical[8-10] studies have shown that carriers of HCM-causing mutations demonstrate signs of diastolic dysfunction, even before a hypertrophic phenotype is observed.

In this thesis, I have studied cellular mechanisms (calcium, sarcomere length, ADP and protein phosphorylation) that, in a complex interplay, regulate relaxation of the heart muscle. Perturbations in these cellular mechanisms may underlie diastolic dysfunction of the failing heart.

CARDIAC CYCLE

The cardiac cycle is characterized by the periods of systole and diastole, which are central to the propelling of blood throughout the four cavities of the heart. Systole is the ability of the heart to contract and eject the blood to the lungs and systemic circulation, while diastole refers to the period of cardiac relaxation and filling.

Cardiac muscle relaxation is the active process that follows contraction, during which the heart decreases its pressure inside the ventricle, fills with blood and prepares for the next cycle of contraction. In the healthy heart the relaxation phase encompasses part of the ejection phase, pressure fall and the initial part of rapid filling.[11] Even if the contraction of the heart is normal, shortening of the relaxation phase will limit proper filling of the ventricle and thereby reduce cardiac output, which can result in HF.

DETERMINANTS OF DIASTOLIC FUNCTION

Myocardial relaxation is intimately related to the ability of the heart muscle to I) decrease intracellular Ca^{2+} levels, II) reduce myofilament Ca^{2+}-sensitivity (i.e. thin-filament deactivation), III) efficiently modulate ATP hydrolysis and IV) detach

cross-bridges (ATP-dependent and mechanical-dependent), which reduces sarcomere stiffness. Moreover, myocardial relaxation is modulated by v) β-adrenergic receptor activation. A detailed description of each is given below (Figure 1).

I. Modulation of myocardial relaxation via intracellular Ca^{2+}-decline

Striated muscle contraction and relaxation is dependent on the modulation of intracellular Ca^{2+}. It takes ~1 mM extracellular Ca^{2+} to increase free cytosolic Ca^{2+} from 0.1 µM during diastole to ~1.6 µM in systole to induce myofilament contraction.[12] Ca^{2+} is highly buffered in cells, which can be attributed to the Ca^{2+} bound to the thin-filament cardiac troponin C (TnC) and the intracellular Ca^{2+} store (sarcoplasmic reticulum, SR), regulated by the SR-Ca^{2+}-ATPase (SERCA).[12] In contrast to skeletal muscle, that almost entirely depends on Ca^{2+} released from the SR to initiate contraction, cardiac muscle depends on both Ca^{2+} entering the cell via the sarcolemma and Ca^{2+} released from the SR (termed Ca^{2+}-induced Ca^{2+}-release) during an action potential.[12-15] In order for relaxation to occur, Ca^{2+} must be removed from the cytoplasm through four Ca^{2+}-transports: 1) uptake of Ca^{2+} into the SR by SERCA; 2) Ca^{2+} removal via the Na^{+}/Ca^{2+}-exchanger; 3) mitochondrial Ca^{2+}-uptake; and 4) Ca^{2+} removal by the sarcolemmal Ca^{2+}-ATPase pump.[16]

Impaired Ca^{2+}-reuptake/removal by one or several of these regulators will increase diastolic [Ca^{2+}], resulting in a state of permanent cross-bridge activation and limited myocardial relaxation.[17]

II. Modulation of myocardial relaxation via myofilament Ca^{2+}-desensitization (thin-filament deactivation)

Myofilament activation and contractility depend on the interaction between the thin actin and thick myosin filament. This interaction is initiated upon electrical activation of cardiomyocytes and the resulting increase in cytosolic [Ca^{2+}]. It has been suggested that the myofilaments oscillate between three biochemical equilibrium transitions, reflecting different interactions between actin and myosin termed the blocked (B-state), closed (C-state) and open (M-state) states of thin-filament regulation.[18,19] In the B-state Ca^{2+} is not bound to cTnC and tropomyosin sterically blocks the myosin-binding sites on actin. The B-state equilibrium is associated with a weakly bound state of cross-bridges. In the C-state, Ca^{2+} binds to cTnC, which changes conformation of the troponin complex and results in a ~25° movement of tropomyosin on the thin filament, thereby exposing most of the myosin-binding sites on actin.[19,20] However in the C-state the myofilament is not yet activated as non-tension-generating cross-bridges are still weakly-bound to actin. The C-state is characterized by intermediate thin-filament activation. The third and final state, the M-state, involves the strong-binding of tension-generating cross-bridges that induces an additional ~10° movement of tropomyosin on the actin filament, resulting in myofilament contraction and force development.[19,20] Muscle relaxation depends on the equilibrium between the 3 states of thin-filament activation.

Disease-mediated changes in myofilament properties may shift the equilibrium to a more activated state (C- or M-state), and thereby increase accessibility of myosin-binding sites on actin, which would increase myofilament force development and limit cardiac relaxation. Defective myofilament proteins as a result of HCM gene mutations may directly impair regulation of muscle contraction. Evidence from genetic mouse models and *in vitro* experiments indicates that HCM sarcomere mutations sensitize myofilaments to Ca^{2+}.[4,21-23] The sensitizing effects of HCM mutations will increase the Ca^{2+}-buffering capacity[21] and affect diastolic function. In Chapter 3 we performed a comprehensive study on a large set of human HCM samples to identify whether high Ca^{2+}-sensitivity is a hallmark of human HCM at low and high sarcomere length. In Chapter 6 we investigated if mutant proteins alter the accessibility of myosin-binding sites on actin.

III. Modulation of myocardial relaxation via efficient ATP hydrolysis of Ca^{2+}-handling proteins

Ubiquitous to the majority of energetic processes in living cells is the enzyme-catalyzed breakdown of phosphoryl groups as a molecular energetic source. A highly relevant reaction is the hydrolysis of ATP into ADP and inorganic phosphate (P_i). Because Ca^{2+}-reuptake by the SR is an ATPase driven reaction, a decline in the rate of ATP breakdown will limit Ca^{2+}-decline in the cytosol. This may result from decreased availability of ATP and/or elevation of ADP/P_i, which will reduce the free energy released from ATP hydrolysis (ΔG_{ATP}). This thereby depresses the activity of SERCA and other sarcolemmal pumps, and delays cytosolic Ca^{2+}-decline, contributing to Ca^{2+}-overload.[24] Again, this will lead to a permanent state of cross-bridge activation during the diastolic phase due to high diastolic Ca^{2+}. In Chapter 5 we measured Ca^{2+}-transients of intact rat cardiomyocytes following elevation of ADP to assess alterations of Ca^{2+}-handling properties.

IV. Modulation of myocardial relaxation (ATP-dependent and mechanical-dependent)

ATP-induced detachment of myosin from actin

At the myofilaments, ATP is the energy-producing reaction whereby ATP hydrolysis (chemical) powers the interaction and sliding of myosin on actin, in order to generate force and motion (i.e. mechanical work). Even though the energy released from ATP hydrolysis is the driving force for myosin-actin interaction and cross-bridge cycling, cross-bridge detachment is independent of the hydrolysis of ATP. Indeed, myosin detachment from actin occurs upon ATP binding to myosin (after ADP has diffused from the nucleotide-binding site of myosin). As little as 0.1 mM ATP is sufficient for cross-bridge detachment and sarcomere relaxation.[25] The relatively high myocardial *in vivo* ATP levels in the healthy heart (8-11 mM) and various cardiac pathologic conditions (7-10 mM)[26-28] thus favour efficient myofilament relaxation.

However, supra-physiological [ADP] (≥250 μM) has been shown to reduce myofilament shortening velocity and generate rigor tension in cardiac muscle from animals by reducing the rate of cross-bridge detachment.[29,30] *In vivo* myocardial ADP levels ranging from 10-50 μM in healthy and of 40-140 μM in diseased hearts[26-28] have been reported in animal models. Elevated ADP levels may thus represent a cause of diastolic dysfunction. To test this hypothesis, in Chapter 5 we performed a comprehensive molecular, cellular and whole organ study to dissect the physiological levels of ADP and Ca^{2+} necessary to impair myofilament function.

Mechanical-induced detachment of myosin from acting

Actomyosin cross-bridge detachment is not solely dependent on ATP-binding. There is theoretical[31,32] evidence that supports a mechanical detachment of cross-bridges, where up to 80% of attached cross-bridges mechanically detach from actin upon stretch. Indeed, rapid shortening and re-lengthening procedures in muscle showed that myosin can be mechanically detached from actin.[33,34] Such mechanical cross-bridge detachment would be favored by high filling of the ventricles during diastole. One may hypothesize that conditions which decrease the detachment rates of myosin, impose rigor stiffness and limit muscle lengthening. In Chapter 5 these concepts were directly tested by applying a protocol of a rapid shortening and relengthening procedure to evaluate how sarcomere stiffness is affected by physiological levels of ADP and Ca^{2+}. In Chapter 6 we used the concepts which were established in Chapter 5 in human cardiomyopathy samples to evaluate if cross-bridge rigor stiffness is altered in sarcomere-mutation positive HCM.

V. Modulation of myocardial relaxation via β-adrenergic receptor activation

Regulation of intracellular Ca^{2+}-levels is directly associated with sympathetic activation of the β-adrenergic receptor pathway as occurs during increased cardiac stress (i.e. exercise). Upon stimulation of the β-adrenergic receptors by catecholamines, cyclic AMP (cAMP) levels are elevated[35,36], resulting in activation of the catalytic subunit of protein kinase A (PKA)[37-43]. PKA mediates positive inotropic, lusitropic and chronotropic responses via phosphorylation of a multitude of factors including Ca^{2+}-handling proteins[44-46] and the myofilament proteins cardiac troponin I (cTnI)[43,47], cardiac myosin-binding protein C (cMyBP-C)[48-50] and titin.[51] β-adrenergic receptor activation accelerates Ca^{2+}-reuptake to the SR via reductions in myofilament Ca^{2+}-sensitivity[40,41,52-54] and increased SERCA activity, which in concert positively modulate myocardial relaxation. Along these lines, the Ca^{2+}-sensitizing effects of the HCM-associated troponin T (I79N, F110I) mutations, and the Ca^{2+}-sensitizing drug EMD 57033, increase the Ca^{2+}-binding affinity ("sticky myofilaments").[55,56] This delays Ca^{2+}-reuptake during diastole and increases end-diastolic Ca^{2+} and makes diseased hearts more susceptible to lethal arrhythmias.[55,56] In Chapter 3, we studied the myofilament function of human HCM samples before and after treatment with exogenous PKA to reveal if myofilament defects are caused by the mutant protein or by a secondary

disease-related reduction in PKA-mediated protein phosphorylation. Moreover, in Chapter 6 we investigated if PKA-phosphorylation deficits alter accessibility of myosin-binding sites on actin, which are predicted to modulate myofilament Ca^{2+}-sensitivity.

FRANK-STARLING RELATIONSHIP – A REGULATOR OF DIASTOLIC FUNCTION

The ability of the heart to adjust the force of its contraction in response to the dynamic changes in ventricular filling forms the basis of the Frank-Starling Law. Ventricular filling regulates the relation between length and tension, thereby controlling ventricular contraction and ejection.[57,58] An increase in muscle fiber length as a result of increased filling during diastole, enhances the maximal force generating capacity and sensitivity of myofilaments to Ca^{2+}, leading to increased force development during ventricular contraction.[57,58] In other words there is a direct relation between sarcomere length and myofilament sensitivity to Ca^{2+} ions, such that more force is generated at a given concentration of Ca^{2+} when sarcomere length is increased. The term 'myofilament length-dependent activation' describes the length-dependent properties of the myofilaments.[57] Clinical observations have shown that HFpEF[59-61] and end-stage HF[62,63] patients have limited capacity to recruit the Frank-Starling reserve. Impaired length-dependent activation is also observed in membrane-permeabilized end-stage human cardiomyocytes.[64]

It has long been appreciated that β-adrenergic receptor activation induces a potent inotropic response of the heart. At the cellular level, length-dependent activation was shown to be positively regulated by isoproterenol, a β-adrenergic receptor agonist[65-67]. Notably, in HF patients β-adrenergic receptors and PKA are down-regulated, while protein kinase C (PKC) is over-expressed[68]. Length-dependent activation can be modulated by protein phosphorylation. Hence, reduced PKA-mediated phosphorylation and increased PKC-mediated phosphorylation may underlie the blunted length-dependent activation which is observed in HF.

A limited Frank-Starling reserve has been observed in HCM patients.[69] This is supported by recent studies in human[9,10] and mice[7] with HCM that clearly show defects of cardiac relaxation that precede the occurrence of left ventricle hypertrophy. The diminished capacity to relax, decreases diastolic filling and limits the effects exerted by length increases, i.e. the Frank-Starling reserve is not used. Indeed, a restricted Frank-Starling reserve has been observed in HCM mutation carriers and transgenic mice models with severe left ventricle hypertrophy.[10,70-72] Data on length-dependent activation in transgenic mouse models harboring HCM mutations have been conflicting and reported either reduced or preserved length-dependent activation.[52,73-75] Findings in mouse models are sometimes difficult to extrapolate to human due to differences in myosin-heavy chain (MyHC) background: the α-MyHC isoform is predominant in mouse ventricles, whereas β-MyHC is the predominant form in the human adult ventricle. A recent study by Ford and colleagues[75] in mice expressing the troponin T R92L mutation in the physiological (mouse-like) α-MyHC

background demonstrated preserved length-dependent Ca^{2+}-activation. Interestingly, length-dependent activation was impaired in mice harboring the R92L mutation in a human-like β-MyHC background.[75] These studies emphasize the importance of myofilament studies in cardiac samples from human HCM patients. Therefore, in Chapter 3 we conducted a large study in human HCM samples to assess if impaired length-dependent activation is a common feature of human HCM, and if this is related to reduced PKA-mediated phosphorylation of myofilament proteins. Moreover, in Chapter 4 we investigated if high PKC target-site phosphorylation (Thr143) of cTnI regulates length-dependent activation.

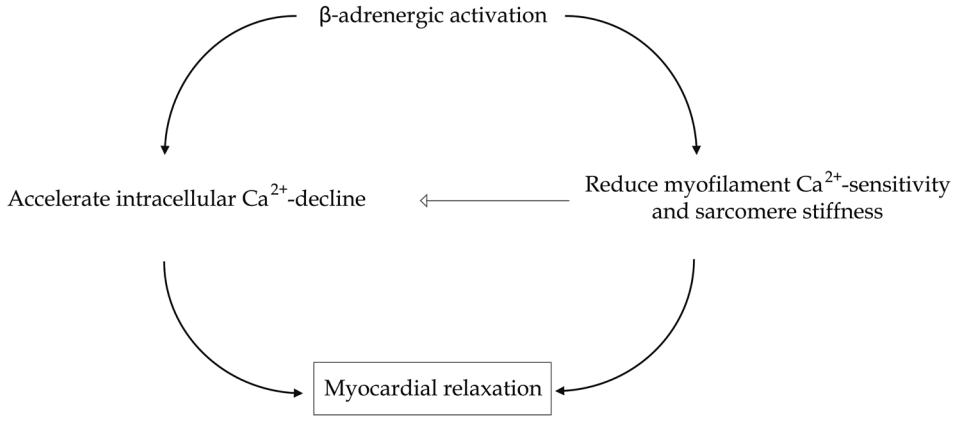

Figure 1. Schematic drawing of the determinants contributing to myocardial relaxation.

AIM OF THIS THESIS

As discussed above, myocardial relaxation is regulated through interacting mechanisms such as the rate of intracellular Ca^{2+} decay, myofilament Ca^{2+}-desensitization, ATP hydrolysis, the rate of cross-bridge cycling, ATP-dependent and mechanical cross-bridge detachment and post-translation modulation (Figure 1). The aim of this thesis was to dissect the cellular alterations related to myocardial relaxation that may contribute to impaired diastolic function in heart failure.

THESIS OUTLINE

Part 1. Regulation of length-dependent activation, the cellular basis of the Starling Law of the Heart

In Chapter 2 an historic perspective of muscle function is provided. Herein a century of scientific research is "revived" from the heart's fundamental protein constituents (contractile proteins), to their assembly in the muscle (the sarcomeres) culminating in an overview of the synergistic events that underlie the Frank-Starling mechanism. As the Frank-Starling Law is a vital mechanism for the healthy heart, it is of utmost importance to understand its mechanical basis to develop and optimize therapeutic strategies to rescue the failing human heart. It is my personal belief that much can be gained by understanding the Frank-Starling relation at the cellular and whole organ level, so that we can tackle the pathophysiologic mechanisms underlying heart failure.

In Chapter 3 we conducted a comprehensive study on a large set of human HCM samples to identify whether length-dependent activation provides a unifying explanation for impaired diastolic function in HCM caused by thick-and thin-filament protein mutations. Measurements in membrane-permeabilized single cardiomyocytes isolated from sarcomere mutation-positive HCM, sarcomere mutation-negative HCM and non-failing donors were performed. To provide direct proof that mutant protein is responsible for the observed effects, troponin exchange experiments with exogenous recombinant wild-type troponin were performed in a unique HCM sample carrying a homozygous troponin T mutation. Finally, myofilament protein phosphorylation was determined using a combination of Pro-Q Diamond/Sypro Ruby staining and phospho-specific antibody targeting. Myofilament Ca^{2+}-sensitivity, phosphorylation background and length-dependent activation mechanisms were studied to understand if determinants of diastolic function are affected by gene mutations in sarcomeric proteins.

In Chapter 4 the effects of cTnI threonine 143 (Thr143) phosphorylation by PKC on length-dependent activation were studied. Troponin exchange experiments were performed in membrane-permeabilized human cardiomyocytes. Human recombinant wild-type troponin and troponin containing pseudo-(de)phosphorylated cTnI at Thr143 were exchanged in non-failing donor and failing cardiomyocytes. Because increased PKC-mediated phosphorylation of Thr143 has been reported in HF models, we investigated if phosphorylation of Thr143 modifies myofilament length-dependent activation in human cardiomyocytes.

Part 2. Myocardial ADP accumulation and contribution of cross-bridge formations to diastolic dysfunction

In Chapter 5 we analyzed the effects of myocardial ADP accumulation as a contributor to diastolic dysfunction. In this study two main determinants of myocardial relaxation were tested, including processes that affect the rigidity of the myocardial wall and the rate of myocardial relaxation. Measurements in membrane-permeabilized single cardiomyocytes isolated from human failing hearts were performed in the presence of ADP (in the absence of Ca^{2+}), in the presence of Ca^{2+} (in the absence of ADP) and in the presence of Ca^{2+} in concert with ADP. Also, the contribution of cross-bridges (actin-myosin interaction) relative to titin was studied with conditions mimicking the *in vivo* situation. We investigated if and how cytosolic ADP accumulation, in concert with diastolic [Ca^{2+}], may underlie impaired diastolic performance.

In Chapter 6 we studied if ADP-stimulated force development (without Ca^{2+}) can be used to reveal changes in actin-myosin blockade (i.e. accessibility of myosin-binding sites on actin) in human cardiomyopathy cardiomyocytes. Cardiac samples from HCM patients, harboring thick- and thin-filament mutations, and idiopathic dilated cardiomyopathy, were compared with sarcomere mutation-negative HCM and non-failing donors. Also, experiments were performed in the presence of Ca^{2+} and force development and stiffness was analyzed. We investigated if the equilibrium of the thin filament activation states is disrupted in human cardiomyopathies, and may contribute to impaired diastolic function.

Part 3. Summary, Conclusions & *Curriculum Vitae*

Chapter 7, Summary, Conclusions & Future Perspectives
Chapter 8, *Curriculum Vitae* of the PhD candidate

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2

HISTORICAL PERSPECTIVE ON MUSCLE FUNCTION: THE FRANK-STARLING LAW

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Historical perspective on heart function: the Frank-Starling Law

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"It had long since come to my attention that people of accomplishment
rarely sat back and let things happen to them.
They went out and happened to things."

Leonardo da Vinci

HISTORICAL MUSCLE PERSPECTIVE

No other organ has inspired so many writers, poets and songwriters as the heart. It is the symbol of love and passion and was once believed to be the place where the soul resides. Even looking at it with cold, unromantic eyes of science, one can appreciate the beauty of this remarkable organ and the circulation attached to it. (with permission Diederik Kuster)

THE HEART

The heart and its vessels, comprises one unique – cardiovascular - system that is responsible for the motion of blood throughout the body.[1] William Harvey's 1628 publication *"Exercitatio anatomica de motu cordis et sanguinis in animalibus"* (On the motion of the heart and blood in animals) showed for the first time: 1) *"that the blood moved in a ceaseless stream, as it were in a circle"* and 2) *"that the heart is the great propelling power"*.[1] Although the anatomy of the heart was well known to physicians at the time of Harvey, namely the existence of four cavities divided by an "impermeable" septum and valves that prevented backflow of material, it was however generally accepted that the heart was *"a generator of vital spirits, and of heat"* and that the propelling of blood was an *"act of inspiration, and its flow to any part of the body determined by special excitation"* (Figure 1).[1]

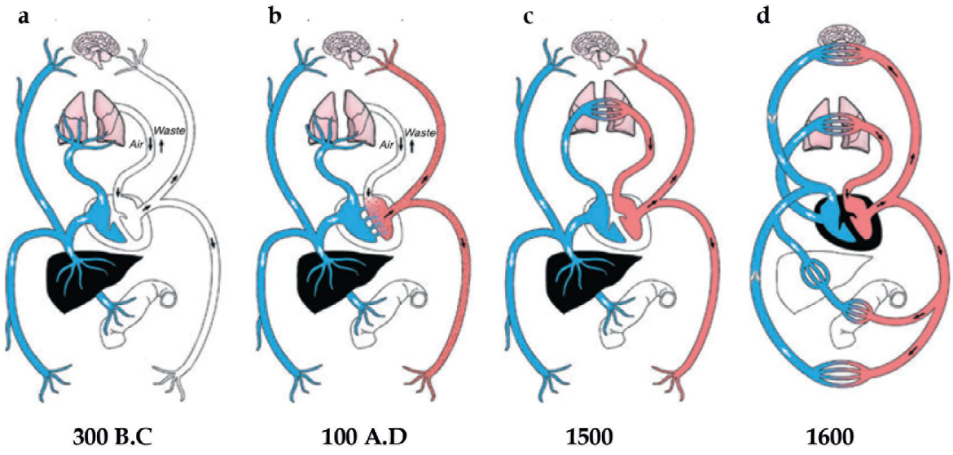

Figure 1. A schematic overview of the cardiovascular system over time. A. Veins (blue) and arteries (white) are separate. Veins transport blood, in opposition to arteries that transport air. B. Arteries (red) transport blood from right side of the heart, after it passes through invisible pores in the septum. C. Establishment of the pulmonary circulation that transports blood through the lungs to the left side of the heart, and the liver was the source of veins and the propelling power of blood. D. Harvey's view of the cardiovascular system. (Figure adapted from Aird WC, 2011[2] with permission John Wiley and Sons).

As we look back to ancient times it is thus obvious to understand why the heart has captured the imagination of so many people. The heart is the only organ whose function we can assess at the body's surface. Can you imagine the admiration our ancestors felt when feeling their heart beat after a threatening or a positive emotional situation? It was only natural to them that this *"generator of vital spirits, and of heat"* was emanating some powerful spirit or emotion, which they could feel instantly in their chests via the acceleration of its beat.

The anatomy of the heart, as it was known before William Harvey in 1600, remains almost unaltered today. Four chambers are observed: two superior atria, right and left atrium; and two inferior ventricles, the right and left ventricle. A transverse inter-dividing septum separates the right from the left side. Veins connect the right and left atria, the venae cavae and pulmonary veins, respectively; in opposition to arteries that connect to the right and left ventricles, the pulmonary artery and the aorta, respectively. Semilunar valves both at the pulmonary artery and aorta prevent backward motion of blood into the right and left ventricle, respectively. In addition, atrioventricular or cuspid valves (tricuspid, the right valve; bicuspid or mitral, the left valve) prevent backflow of blood from the ventricles into the atrium during contraction.

Cardiac cycle

The cardiac cycle is characterized by the periods of systole and diastole. In brief, systole comprises isovolumetric contraction and ejection, while diastole refers to the period of isovolumetric relaxation and filling. The cardiac cycle periods are central to the propelling of blood through the four cavities of the heart. Notably, the concepts of systole and diastole did not always have the same meaning. As Brutsaert and Sys[3] describe:

> *This is not surprising, as the meaning of most scientific words may be subject to modification as long as the experiments, observations, theories, and concepts to which they refer have not been clearly elucidated. Due to the confusion about the meaning and delineation of systole and diastole, Edgren, as early as 1889[4], insisted that both these terms ought to be banished altogether from medical literature and ought to be replaced simply by contraction and relaxation, respectively. (Brutsaert and Sys[3] with permission Physiological Reviews)*

William Harvey was the first to correctly define diastolic (relaxation) and systolic (contraction) phases of the heart, as it was previously believed that diastole reflected the contraction period (*'the dilatation of arteries at each pulse'*).[1]

> *Whence the motion which is generally regarded as the diastole of the heart, is in truth its systole. And in like manner the intrinsic motion of the heart is not the diastole but the systole. (William Harvey[1]. Publisher and author could not be located)*

It took 300 years before Carl J. Wiggers[5,6] consolidated the meanings of systole and diastole that survive with minor modifications up to day; the clinical division of systole and diastole is based upon the closure and opening of the mitral and aortic valves.[3]

"The working capacity of a pump is measured by its output"

In the human body the heart is the main responsible for the supply of blood perfusion with vital nutrients and gases to organs and tissues. It is thus only natural that the study of the heart parallels that of a pump, which moves fluids by mechanical action. The pump function of the heart is used to assess cardiac function in humans. As stated by Patterson and Starling, *"the working capacity of a pump is measured by its output."*[7] In the heart, cardiac output (CO) is the interdependence of blood volume ejected by the ventricles per contraction/heart beat - stroke volume (SV) - and the heart beat frequency - heart rate (HR) - occurring in one minute [CO = SV x HR].

Harvey's description of the motion of blood greatly advanced the thinking of nineteenth-century physiologists[8-11] and remains remarkably accurate to day. This was summarized by Patterson and Starling[7] one hundred years ago (1914),

> *At the beginning of ventricular systole the ventricular muscle passes rapidly from a condition of relaxation to one of contraction. The auriculo-ventricular valves close at the very commencement of the ventricular contraction. The contraction of the muscle, or rather the rise of contractile stress, then proceeds for a time isometrically [this phase is not entirely isometric, but is isovolumic (volume remains unaltered), as individual fibers undergo changes in length (shortening and/or lengthening)[12,13]], while the pressure in the ventricle is rising to the pressure in the aorta. When the pressure rises to this point the aortic valves open and the muscle fibres are able to shorten. The pressure, however, continues to rise for a time and then falls […]. The contraction then ceases and relaxation begins with coincident rapid fall of pressure in the ventricle below that in the aorta, which causes a closure of the aortic valves. From this time forward the relaxation of the ventricular muscle [ensues] [...] in order that the ventricular muscle may lengthen, the ventricles have to be distended by the inflow of fluid, though the pressure necessary to distend them is almost negligible. But whereas in the case of the skeletal muscle (under ordinary experimental conditions) the tension exerted by the extending force is the only factor of importance, in the heart the rate of application of the extending force, i.e., the rate of inflow of the blood, is still more important than the tension exerted by it on the walls of the ventricle. The ventricles cannot dilate unless fluid enters them, so that they may be, so to speak, 'after-loaded' at every stage of their relaxation. If blood therefore does not enter the heart, the ventricles do not dilate at all during diastole and may become smaller and smaller with each systole until they are empty. (Patterson and Starling[7] with permission John Wiley and Sons)*

Identical processes occur on the right side of the heart, differing mainly from the left side by: 1) thinner right ventricular wall, associated with 2) lower pressures,

and 3) lower resistance to outflow – afterload - into the 4) pulmonary trunk (pulmonary circulation).[1,14,15]

THE FRANK-STARLING LAW OF THE HEART

The ability of the heart to adjust the force of its contraction in response to changes in ventricular filling (end-diastolic volume, EDV) forms one of the main pillars of muscle physiology. Ventricular filling sets the relation between sarcomere length and tension development, and determines the degree of muscle shortening, which thereby regulates ventricular contraction and ejection.

The observation that cardiac muscle contraction is the interdependent relation between tension development, heat production and the extent of muscle shortening, as a function of the initial length of a muscle fiber, was first described for skeletal muscle by Magnus Blix[16] and von Kries[17,18]. They proposed that the energy of a contracting muscle is a function of its length and that its shortening velocity is directly proportional to the "afterloaded" condition (e.g. shortening velocity increases as afterload decreases). The latter findings were later applied by the German physiologist Otto Frank[11,19] to the heart, who proposed that the developed pressure was directly proportional to the initial diastolic tension.

> *Length and tension changes in skeletal muscle correspond to changes in volume and pressure [in the heart]. By measuring these values and establishing their time relationships, one is able to examine the play of forces in the entire heart. (Otto Frank[19] with permission Elsevier)*

> *[…] if initial tension (or filling) is continuously increased by means of raising the blood-reservoir, the ejected volume increases until the initial tension has become equal to the pressure in the arterial system. (Otto Frank[19] with permission Elsevier)*

However, Otto Frank's experiments were inconclusive to whether an increase in the force of contraction was related to the initial diastolic tension or length of muscle fibers.[20] Ernest Starling and colleagues[7,20,21] later showed that *"it is length rather than tension which determines the energy of contraction"* and therefore stated:

> *There can be no question therefore that it is the volume of the ventricles at the beginning of contraction rather than the pressure within their cavities, which determines the amount of energy set free during the contraction. (Ernest Starling[22] with permission Nature Publishing Group)*

> *This relation between the length of the heart fibre and its power of contraction I have called 'the law of the heart' [...] The law of the heart is therefore the same as that of skeletal muscle, namely that the mechanical energy set free on passage from the resting to the contracted state depends [...] on the length of the muscle fibers [...]. The greater the length of the fibre, and therefore the greater amount of surface*

> *of its longitudinal contractile elements at the moment when it begins to contract, the greater will be the energy in the form of contractile stress set up in its contraction (Patterson, Piper and Starling[20] with permission John Wiley and Sons)*

Accordingly, the *"Law of the Heart"* or the "Frank-Starling relation", reflect the ability of the heart to adjust the force of its contraction, in response to volume changes in venous return.

Thus the Frank-Starling relation explains beat-by-beat adjustment of cardiac output by both sides of the heart (*"events on the right side reflect those in the left side of the heart, unless failure is present"*[7,23]) and pathological conditions that directly affect the Frank-Starling response (e.g. diastolic dysfunction) represent life-threatening situations. As we will see below, the Frank-Starling relation is inherently associated with its fundamental constituents - cardiac muscle cells. Notably, in 1914, Ernest Starling already desired to understand the pathological conditions that lead to heart failure (HF):

> *If there is increase inflow followed by optimal ED [end-diastolic] dilatation [...] beyond this point, the heart dilates pathologically and performance falls of. (Patterson and Starling[7] with permission John Wiley and Sons)*

> *Within physiological limits the larger the volume of the heart, the greater are the energy of its contraction and the amount of chemical change at each contraction. (Ernest Starling[22] with permission Nature Publishing Group)*

> *Fatigue of the heart may go on to heart failure. This occurs when the dilatation, which is the mechanical result of unchanging inflow and failing outflow and is the automatic means of regulating outflow to inflow, proceeds to such an extent that the tension of the muscle fibers becomes increasingly inadequate in producing rise of intracardiac pressure. (Patterson, Piper and Starling[20] with permission John Wiley and Sons)*

Frank-Starling Law of the heart, and its place in history

Several concepts initially scrutinized this Law, including 1) the originality of Frank and Starling's ideas, namely that for several authors they rediscovered the relation of increased contractile power when the muscle size is increased;[24,25] and 2) the objections to the clinical applicability of the concepts of the Frank-Starling relation over the last century.

1. For instance, in 1936, Hans Gremels[26] was the first to speak about the *"Frank-Starling Law of Cardiac Work"*[19], advancing what some previously noted as some sort of a "flaw":

> *The so-called Law of the Heart, which Starling rediscovered ten years later [than Frank][26] and that is linked with Starling's name especially in the English literature (Otto Frank[19] with permission Elsevier)*

In addition, it is still open for debate as to whom the credit for the Frank-Starling relation should be given.[24,25] Abe Guz remarked in the introduction of the "Ciba Foundation Symposium 24 - Physiological Basis of Starling's Law of the Heart"[27] in 1973:

> *I have been asked why the title of this symposium was not 'The Physiological Basis of the Frank-Starling Relation of the Heart'. If we were to give credit in full, we would have to call it the 'Hales[28]-Haller[29]-Müller[30]-Ludwig[31]-Roy[32,33]-Howell[34]-Donaldson[34]-Frank[11]-Starling[22] relation (Abe Guz[27] with permission John Wiley and Sons)*

But earlier Chapman and Mitchell[35] had argued that:

> *In any event, Starling's work represents a convergence of various German and British intellectual forces [...] It was his genius that brought these various forces together in meaningful synthesis and it was largely his generalizations that provoked, and still provoke, highly constructive exchanges on the subject of myocardial control between biomedical scientists everywhere. (Chapman and Mitchell[35] with permission Prof. Jere Mitchell)*

It is my personal belief that Ernest Starling stated it as a "Law" and this view was expressed by Stephen Hawking (in his book the *Grand Design*) who stressed the importance of Nature's Law's to the success of mankind: *"Today most scientists would say a [L]aw of nature is a rule that is based upon an observed regularity and provides predictions that go beyond the immediate situations upon which it is based."*

A Law requires the prediction of phenomena or events that go beyond the mere observation and can be measured and validated by others.

2. The impact of the Frank-Starling Law gained wide acceptance after Starling presented his concepts in 1915[22] and 1920[36]. This historical period is greatly illustrated by Chapman and Mitchell[35], for which the author will present a short summary with authors' quotes (Chapman and Mitchell[35] with permission Prof. Jere Mitchell).

Starling's concepts rapidly diffused from the 1920s till the 1940s, gaining wide appreciation in particular to describe HF. Later on, his work was subjected to intense scrutiny, mainly because during exercise it was stated "*that with increasing work loads cardiac size increased; but the cardiac size was never as much as would be expected in view of the associated increase in cardiac output per minute*"[35] and that it could not explain cardiac failure[37] as it was "*found that injection of human albumin* [albumin temporarily increases blood volume and pressure] *into normal subjects and into patients with circulatory failure produced a rise in right atrial pressure but did*

not affect cardiac output in a consistent manner"[35]. However, in the 1950s Starling's concepts consolidated as one of the most important mechanisms of the heart, if not perhaps the most important. First by the work of John McMichael[38] where "*he published three theoretical curves [...] relating stroke work with filling pressure for the normal, the hypodynamic and the hypertrophied heart. The suggestion was that all three curves might be seen in a single heart, depending on experimental conditions*"[23] and secondly by the "curves hypothesis" from Sarnoff and Berglund[39]:

> *Many investigations of this matter in the presence of a complete circulation have been either inadequate or misleading largely because of the following: (1) stroke volume or cardiac output and not stroke work has been used as the measure of the "energy of contraction"; (2) attempts have been made to correlate right sided filling pressure with left ventricular stroke work; (3) attempts have been made to correlate filling pressure with ventricular work per minute instead of per stroke, and (4) perhaps most important, it has not been generally appreciated that a single Starling curve cannot always satisfactorily explain the observed phenomena; for any given heart there is a series or family of curves. (Sarnoff and Berglund[39] with permission Wolters Kluwer Health, Inc)*

This line of thought has been since then further reevaluated and confirmed by experimentation with the final conclusion that the "[Frank-*]Starling's law of the heart is applicable to man"*[35].

CARDIAC RESERVE MECHANISMS

The normal heart is able to maintain or increase its output by several mechanisms that are mutually related: 1) recruitment of the Frank-Starling reserve; 2) increasing heart rate, which enhances the force of contraction via increases in the force-frequency relationship or Bowditch effect; 3) increases in the peak force generated during a contraction - positive inotropic response or enhanced contractility (e.g. hormones); and 4) elevations of afterload and the Anrep effect. Following an afterload elevation (e.g. vascular resistance) there is a rapid increase of end-diastolic volume that increases contraction (Frank-Starling). This initial rapid response is followed by a progressive and time-dependent (1-2 minutes) enhancement of contractility, which is independent of length-alterations and allows the ventricle to recover towards its normal volume.[40]

Cardiac inotropic reserve

Other factors, such as hormones affect the pump function of the heart.[36,40,41] In 1891 Erik Johansson observed in dogs that stimulation of the splanchnic nerves raises arterial blood pressure.[42] Later, Arno Lehndorff[43] investigated a two-part rise of blood pressure confirming that the initial rise was due to vasoconstriction of the splanchnic area, and the second due to increased heart rate. Four years later, Thomas R. Elliott[44] demonstrated that the second rise was caused by secretion of adrenalin. Then Glen

von Anrep[40] established the link between accelerated heart rate and the secretion of adrenalin described by Thomas R. Elliott[44].

> *In this paper I give an account of some observations with regard to this, made at the suggestion of Prof. Starling [...] I have shown that the acceleration [of the heart beat] is due to the increased production of adrenalin. (Glen von Anrep[40] with permission John Wiley and Sons)*

These findings were extended by Ernest Starling[36] who noted that secretion of adrenalin dramatically increases *"the energy available at each contraction."*, i.e. the modern positive inotropic effect introduced in 1904 by Theodor W. Engelmann[45]. Charles M. Gruber[46,47] observed similar findings in skeletal muscle, who showed that epinephrine exerts potent positive inotropic effects.

The Anrep effect or slow force response

In 1912, Glen von Anrep[40], working in Ernest Starling's laboratory, observed that if arterial resistance was abruptly elevated, the end-diastolic volume first increased but then after several minutes an increased contractility occurred allowing ventricular volume to return to baseline[40]. Anrep showed that this phenomena was especially marked after adrenaline administration.[40]

> *We may conclude therefore that a mechanical rise of arterial pressure, even a very small one, always produces a passive dilatation. If however the blood-pressure be maintained steady at its new height, the heart always reacts in such a manner that it tends to return more or less to its normal volume, whether the change in pressure has been one of diminution or of increase. This reaction of the heart is especially powerful after the administration of adrenalin. (Glen von Anrep[40] with permission John Wiley and Sons)*

Two years later Patterson, Piper and Starling[20] observed the same phenomena,

> *This two-fold effect of a rise or fall of arterial pressure was described by Anrep as an attempt of the heart to return to its proper volume. (Patterson, Piper and Starling[20] with permission John Wiley and Sons)*

but attributed it effect to *"improved nourishment of the muscle"*, suggesting that myocardial metabolism occurs with increased coronary flow when arterial resistance is increased. The authors excluded the increased contractility has the resulting improvement of the contractile state of muscle. Anrep later discarded his own observations in favor of the much simpler explanation proposed by Patterson, Piper and Starling.[35] However, in 1959, it was found by Rosenblueth and colleagues[48] that the effect reported by Anrep was independent of coronary blood flow changes, which was also confirmed one year later by Sarnoff and colleagues[49]. Sarnoff and Mitch called

this the "Anrep effect"[50], and named it the "homeometric autoregulation"[49], because it did not depend on changes in length to distinguish it from the length changes in the Frank-Starling relation, the "heterometric autoregulation". In brief, the Frank-Starling mechanism is regarded as a rapid force response because it depends on fiber length alterations that act at each beat, thence classically operating on a "beat-by-basis"; in contrast to the Anrep effect, a slow force response that is dependent on the contractile state and develops after a few minutes. *In vitro* studies commonly use the term "slow force response".[51]

Parmely and Chuch[52] used isolated papillary muscle to show that stretching induces the Frank-Starling effect followed by a slow increase in inotropic state if the muscle length remained constant. This slow force response has been attributed to slow increases of the Ca^{2+}-transient (in contrast to the Frank-Starling effect that is independent on Ca^{2+}-alterations) due to stretch-induced release of autocrine/paracrine effectors, including angiotensin II, influx of Na^{+} or/and Ca^{2+} via stretch-activated sarcolemmal channels and augmented Ca^{2+} sparks as a result of stretch-induced production of nitric oxide.[51] It is worth considering that the existence of such slow force responses may represent a compensatory mechanism that allows a given change in cardiac work (output) to be accomplished with smaller changes in end-diastolic volume (cardiac size). One can speculate that the second, slow force response compensates for the excessive use, or "fatigue", of the Frank-Starling reserve. In other words, because the heart operates on the ascending limb of the Starling curve (1.8 μm to 2.3 μm sarcomere length; discussed below), enhanced dilation of the heart would impair the maximal force and shortening velocity developed. Thence, the heart, by the existence of sophisticated inotropic responses, efficiently regulates myocardial performance.

STRIATED MUSCLE STRUCTURAL UNIT, THE SARCOMERE

Seventeenth century microscopists Robert Hooke and Antonie van Leeuwenhoek paved the way for the characterization and description of skeletal and cardiac muscle cells.[53,54] Both types of cells have a striated pattern when observed under the optical microscope, and thus are collectively known as "striated muscle". Solely controlled by the central nervous system, skeletal muscle cells are regarded as a "voluntary" muscle, as opposed to "involuntary" cardiac muscle, which is controlled by an intrinsic pacemaker activity under the control of the autonomic nervous system. Notably, the heart is able to maintain its contractility even without stimulation of the nervous system.

> *From this [dog heart-lung] preparation we learn that the heart, freed from all its nervous connections, has the power of automatically adjusting the force and extent of its contractions to the task which is set by the two factors determining its work, viz: the inflow into the heart from the veins, and the resistance offered to the outflow by the arterial pressure.(Ernest Starling[36] with permission BMJ Publishing Group Ltd)*

Striated muscle fibers are composed of small assemblies, called "myofibrils" that contain the contractile components (Figure 2, upper image). They contain a succession of transverse striations that form the fundamental structural unit - the sarcomere (Figure 2, lower image).[55,56] Each sarcomere is delineated by a pair of "Z-lines" (from the German word "*Zwischenscheibe*"; meaning "intermediate disc"[55]). Sarcomeres are characterized by alternate zones of light I (isotropic) bands and dark A (anisotropic) bands.[53,57,58] Each I-band is bisected by a Z-line and consists of thin-filaments, while the central A-band contains both thin- and thick-filaments.[59] The H-band (from the German "*Heller*"; meaning "brighter") in the middle of the A-band solely composed of thick-filament structures.[60] Finally, a dark M-line in the middle of the H-band (so called from the German "*Mittelscheibe*"; meaning "central disc"),[61] is critical for the organization of the thick-filaments in the sarcomere.[62]

Contractile components of muscle

During each heart beat, cardiomyocytes undergo changes in length and load to allow the filling or ejection of blood. The sarcomeres shorten by converting chemical energy into mechanical force to perform work. They contain the contractile proteins that govern muscle contraction and relaxation, and the structural proteins. Myofilament contraction requires the interaction of the thin- (actin-containing) and thick-(myosin-containing) filaments.[64] Force production or/and muscle shortening are the collective sum of all the tension-generating cross-bridges. Regulation of this interaction depends on Ca^{2+} and ATP as well as the regulatory troponin-tropomyosin complex bound to the actin thin-filaments.[65,66] The cytoskeleton forms the scaffold that regulates cell shape, provides mechanical integrity and resistance, and stabilizes the sarcomeric proteins. Importantly this framework mediates biomechanical and biochemical cell signaling that alters gene expression, post-translational modulation and protein synthesis.[67,68]

The sliding filament hypothesis

> *The official date of the 'birth' of the sliding filament theory of muscular contraction is May 22, 1954. On this day the journal Nature published two papers consecutively under the general title: 'Structural Changes in Muscle During Contraction'. The first paper by Andrew F. Huxley and Dr. Rolf Niedergerke[69] was entitled: 'Interference microscopy of living muscle fibres'. The second paper by Dr. Hugh Huxley and Dr. Jean Hanson[70] was entitled: 'Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation'. (Jack A. Rall[71] with permission Springer)*

These papers provided the molecular and mechanical foundations for muscle contraction. Both used high-resolution microscopy to study the structural arrangement of sarcomeres during contraction. They observed that during contraction, the A-band length remained constant, while the I-band changed length as it slid past the A-band.[69,70] These observations signaled the 'birth' of the "sliding filament

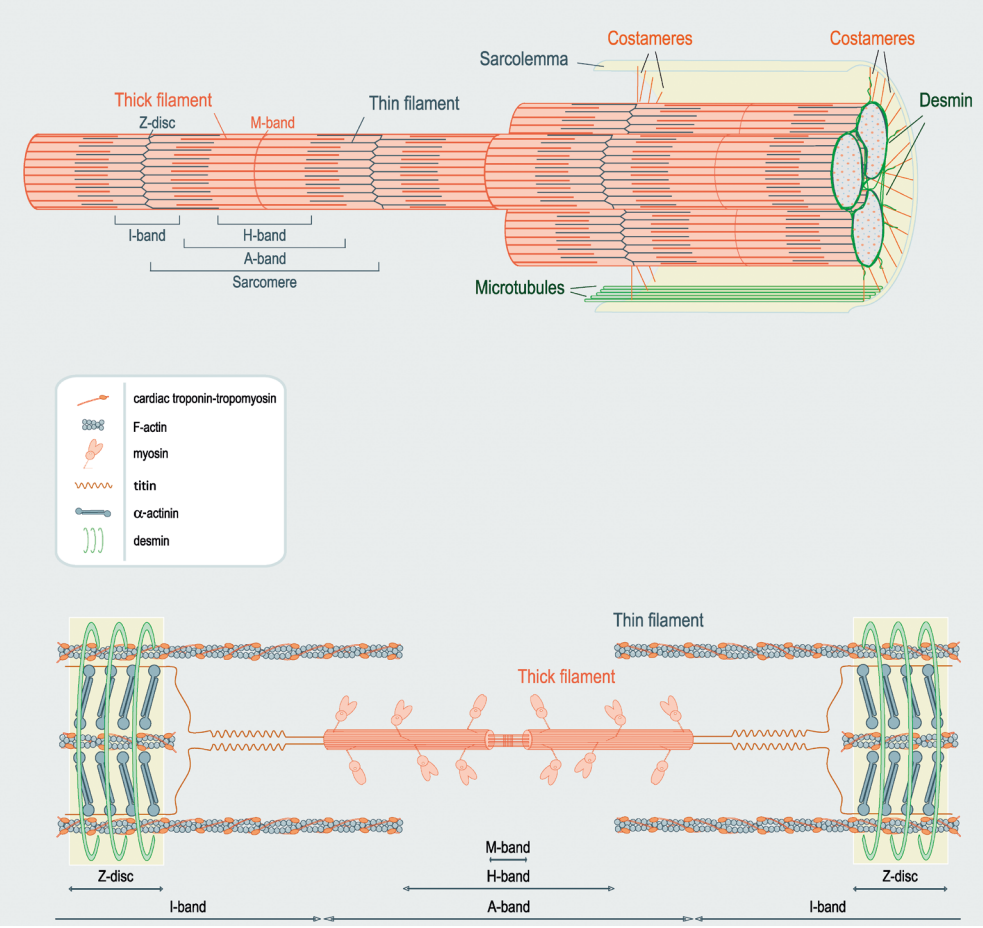

Figure 2. Anatomy of cardiac muscle. The (upper image) illustrates a group of myofibrils connected to the sarcolemma via the costamere network. The (bottom image) shows an individual sarcomere. Note the formation of distinct bands. The components are not drawn to scale. (Figure adapted from Sequeira et al, 2013[63] with permission Elsevier)

hypothesis". In 1957 Andrew F. Huxley[64] proposed that the two sets of filaments interact and overlap forming several individual structures that Hugh E. Huxley termed as "cross-bridges".[72]

> *Bridges are observed between primary [thick-filament] and secondary filaments [thin-filament]. These bridges form part of the primary filaments, and in stretched muscles can still be seen projecting into the space from which the secondary filaments have been withdrawn […] Cross-bridges between primary and secondary filaments are prominent features of all these pictures. (Hugh E. Huxley[72] permission not required)*

The resulting force is the collective sum of all "activated" cross-bridges that pull actin filaments towards the center of the sarcomere. Thick-filaments are organized in hexagonal arrays, and each thick-filament is surrounded by six actin filaments.[73-77] Myosin molecules are packed "tail-to-tail" at the center of the sarcomere in anti-parallel alignment and optimize contact with the actin monomers (Figure 3A).[78,79] Filament movement is directed towards the center of the sarcomere and is entirely determined by the actin filaments on each side of the Z-disc. Relative sliding of the two sets of filaments occurs when actins are "pulled" by myosins.[79] The cross-bridges were originally described as "oar-like"[80] but when their structure was revealed at atomic resolution, the cross-bridges are now known to maintain a fixed angle with respect to the thin-filaments and the rotation is due to the converter domain closer to the base of the cross-bridge[81].

MUSCLE ULTRASTRUCTURE: ELEMENTARY COMPOSITION

Myosin, the major component of thick-filaments

Originally known as "globulin", myosin was defined in 1895 by Otto von Fürth.[83] It was the first sarcomeric protein to be studied. Major advances in its chemistry and localization were performed by Annemarie Weber who showed that the birefringent properties of the A-bands were the direct result of the birefringence of

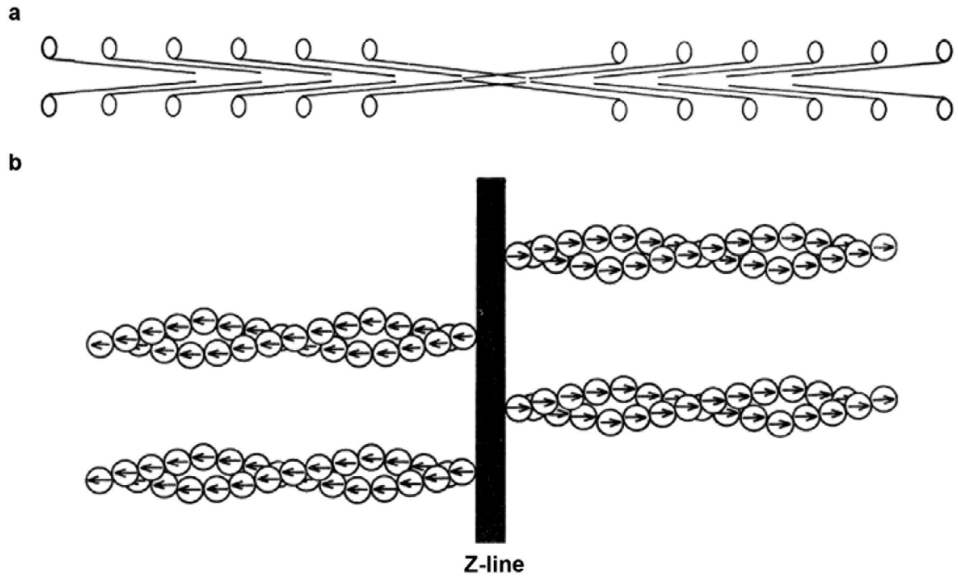

Figure 3. A. Diagram of myosin arrangement in the thick-filament. B. Represents actin molecule polarity pointing away from the Z-line. (Figure adapted from Huxley HE, 1970[82] with permission Royal Society).

"*myosin threads*".[84,85] In 1939, Engelhardt and Liubimova[86] reported that myosin is the main enzyme responsible for the hydrolysis of ATP.

Structurally and each myosin has a long "rod-like" silhouette composed of one heavy chain and two light chains (Figure 4).[87-91] Each heavy chain has a light-meromyosin (LMM), the smaller shorter component, and a heavy-meromyosin (HMM), the larger component.[92,93] These two components can be separated by digestion with papain and/or trypsin using gel electrophoresis (Figure 4).[94-97]

The LMM forms the structure of the thick-filament, while the HMM acts as a "hinge", allowing S1 to move towards actin.[98] This hinge property, propelled by

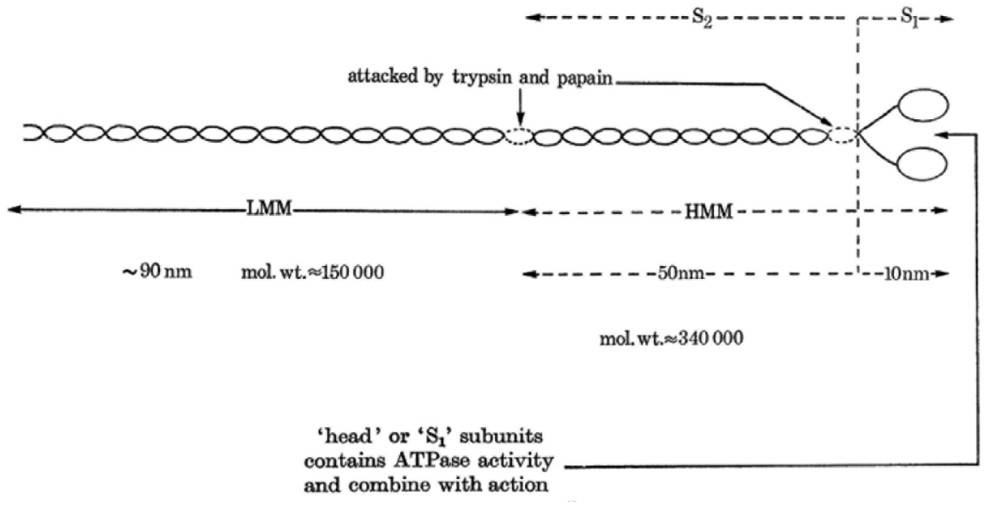

Figure 4. Schematic representation of myosin (Figure adapted from Huxley HE, 1970[82] with permission Royal Society). Here S1 represents the myosin head, S1 and S2 comprise the cross-bridge, and LMM forms the bulk of the thick filament.

the hydrolysis of ATP, governs actin-binding. The HMM is additionally divided into two sub-fragment extractions: sub-fragment 1 (S1)[87,96,97] and sub-fragment 2 (S2)[99]. S1 accounts for 55-60% of HMM[87] and comprises two globular heads containing an actin-binding component and catalytic ATPase activity. S2 separates the rigid LMM from the very flexible HMM.[88]

Actin, the major component of thin-filaments

The main constituent of the thin-filament is actin.[70,100,101] The actomyosin complex was originally budded "myosin B" in 1941 by Banga and Szent-Györgyi[102] to distinguish it from the myosin (called myosin A) based on their different solubility and viscosity properties. Actin was thought to represent a second form of myosin when extracted from muscle, but Brunó F. Straub[103] later found that it does not contain myosin B, but

instead contains an association of proteins called "actin" that combine with myosin (actin + myosin). Myosin B was later called actomyosin, representing the polymerized form of actin and myosin.[103]

Actin exists in two forms. A continuous monomeric strand of globular "inactive" actin (G-actin) spontaneously polymerizes to form the filamentous "active" actin (F-actin), a double-stranded polymer, forming the backbone of the thin-filament.[70,103,104]

In the absence of salts it is present in solution as inactive actin, in the presence of salts as active actin (Brunó F. Straub[104] with permission S. Karger)

In striated muscle, two isoforms are predominantly expressed: α-skeletal actin (*ACTA1*; over 95% present in adult skeletal muscle) and α-cardiac actin (*ACTC1*; over 80% present in the adult heart) that share near 99% homology.[105] Actin assembles into F-actin that serves as a rigid cytoskeleton platform for the binding of troponin-tropomyosin, the thin-filament. Spanning the I-band, the opposing fast growing (barbed) ends of actin filaments are "capped" (blocked) and anchored by a protein called "CapZ"[106] to the Z-disc, as opposed to the slow growing (pointed) filament ends that are capped in the A-band by tropomodulin.[107]

Actomyosin/cross-bridges and ATP

An essential property of biological systems is their ability to convert chemical energy into mechanical energy. It was originally believed, and almost unchallenged, that the primary energy-producing reaction of muscle was lactic acid formation via the usage of glycogen. In 1927 Fiske and Subbarow[108] showed that an *"unstable form of phosphorus (which we shall for the present designate as 'labile phosphorus')"*, i.e., phosphocreatine (PCr), decreased during contraction and was restored upon recovery. Evidence for the latter came in the early 1930s from Einar Lundsgaard in a series of papers[109-111] whose concepts overthrew the lactic acid theory of contraction. He showed that muscles poisoned with iodoacetate resulted in muscle spasm and stiffness (rigor) without lactic acid formation. Lundsgaard concluded that PCr was likely the direct source of contraction based on the observation that PCr content was zero in the poisoned muscles.[109] Together these data triggered the interest in the study of PCr. Nevertheless, experiments performed in the same decade indicated that no enzyme could use PCr as a direct fuel source in muscle.[112] In 1934[112] and 1935[113], Lohmann demonstrated that "creatine kinase" (CK) could breakdown PCr, resulting in the conversion of ADP to ATP:

$$PCr + ADP \rightarrow Cr + ATP$$

The author concluded that ATP breakdown preceded PCr breakdown in muscle contraction, thus providing strong evidence that ATP cleavage is the energy producing reaction of muscle.[112] Today we know that ATP splitting (chemical) powers

the interaction and sliding of myosin on actin, in order to generate force and motion - work (mechanical). CK belongs to a group of energy buffering systems that maintain *in vivo* levels of ATP. CK catalyzes the transfer of phosphate from PCr to ADP regenerating ATP while preventing accumulation of ADP.[114]

One of the most accepted theories explaining the sliding process is the cross-bridge theory, which suggests that the energy released from ATP hydrolysis is the driving force for myosin extension towards actin.[64,69,70] Here, each bridge performs a number of cycles of attachment to and detachment from actin, which is accompanied by changes in myosin orientation and its initial conformation, changing the affinity of myosin's for actin. It has been shown that full ATPase activity of the S1 is greatly stimulated by Mg^{2+} - MgATPase activity.[115-117] When ATP is present, myosin and actin do not interact (step 2, Figure 5).[118] Because myosin-S1 can hydrolyze ATP into ADP and P_i (step 3', Figure 5)[119,120] this process[121] is strongly enhanced by binding to actin (step 4, Figure 5).[81] Moreover, formation of weak-binding cross-bridges occurs. A change of actomyosin conformations occurs (steps 5 and 6, Figure 5) with the release of P_i from the S1 head (step 7, Figure 5) accompanied by strong-binding cross-bridges.[122-124] Force generation and work accompany muscle shortening. ADP is then released (step 8, Figure 5) and a new ATP molecule binds to the actomyosin complex to begin a new cross-bridge cycle (step 1, Figure 5). If ATP is lower than 0.1 mM[125,126], cross-bridges become permanently attached[75] (step 8, Figure 5) and the muscle becomes rigid ("rigor mortis").[118,127] For a detailed summary of events see[128].

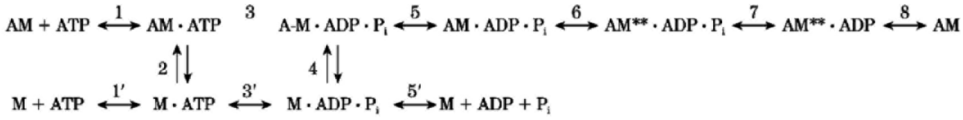

Figure 5. Cross-bridge cycle. (Figure adapted from Gordon et al. 2000[128] with permission Physiological Reviews).

Tropomyosin, a thin-filament associated protein

Tropomyosin (Tm) spans each seven actin monomers. Discovered by Kenneth Bailey in 1946[129] the author proposed that due to its "*analytical and structural similarities [...]* [tropomyosin] *is a species of myosin differing mainly in the length of the polypeptide chain"* and so *"in proposing the present name, we have deemed it desirable to retain the word 'myosin' and to add a prefix which suggests this specific relationship." (Kenneth Bailey[129] with permission Nature Publishing Group)*

Today, tropomyosin is far from being a species of myosin. It has two parallel α-helical chains, overlapping head-to-tail along the thin-filament.

Setsuro Ebashi was the first to recognize the role of tropomyosin in muscle.[130,131] He demonstrated that a new protein with similarities to the tropomyosin described by Kenneth Bailey was required for the ability of the actomyosin complex to be sensitive to Ca^{2+}.[130] This protein ("native" tropomyosin) was the combination of tropomyosin with a new globular protein Ebashi later called "troponin".[132] Troponin exhibited "cementing" (stabilizing) effects on the interaction between actin and tropomyosin,[133-135] and regulated the position of tropomyosin on actin, in a Ca^{2+}-dependent manner.[66,136-139]

Troponin complex, thin-filament associated component

Troponin (Tn) was initially termed by Ebashi and colleagues[133,140] in 1965 as a "tropomyosin-like protein" due to its similarities to the tropomyosin reported by Bailey[141] as follows,

> *viscosity-ionic strength relationship, the binding capacity to F-actin and the sensitivity to trypsin digestion […] the presence of the [troponin] component makes actomyosin tend to relax, or dissociate, if the concentration of free calcium ions are lowered. (Ebashi S. and Ebashi F.[132] with permission Oxford University Press)*

Troponin bound Ca^{2+} and regulated tropomyosin movement on actin.[138] Nevertheless, instead of a single protein exerting multiple roles in muscle contraction, a "flavour" of different troponin subunits was discovered with distinctive structures and functions.[142-150] The troponin complex was separated into 2-4 gel fractions when precipitated under particular conditions, such as the presence or absence of Ca^{2+}, actin, myosin, actomyosin and tropomyosin.[142-150] The identification and clarification of each fraction became a challenge with distinct methodologies producing differing results, including separation of different fractions on gels, different molecular weights and nomenclatures (troponin A, troponin B, inhibitory factor, Ca^{2+}-sensitizing factor, TN-I, TN-T, TN-C, fraction I, II, III and IV).[151] Table 1 below (from Perry and colleagues)[152] illustrates this dilemma.

Several properties of the troponin subunits included components that: 1) were capable of binding Ca^{2+} (troponin C, troponin A, Ca^{2+}-sensitizing factor, troponin fraction III and troponin 4); 2) inhibited actomyosin interactions (troponin I, troponin B, inhibitory factor, troponin fraction II and troponin 2); and 3) interacted with tropomyosin (troponin T, 37,000 component, troponin fraction T and troponin 3). The fourth fraction was suspected to be a contaminant and is omitted from Table 1.[144,150,153]

Based on these data, Hugh E. Huxley in 1972[136] proposed the adoption of the following scheme:

> *There is some divergence about nomenclature, and although I hesitate to intrude into this field, I think everyone might agree that there are advantages to be gained*

Table 1. Components of troponin (Table adapted from Perry et al.[152] with permission Cold Spring Harbor Laboratory Press).

Calcium-Binding Protein	Inhibitory Protein	37,000 Component	References
Troponin A	Troponin B		Hartshorne and Mueller, 1968[142]
Calcium-sensitizing factor	Inhibitory factor	37,000 component	Schaub and Perry, 1969[154]; Wilkinson et al., 1971[155], 1972[150]
Troponin 4	Troponin 2	Troponin 3	Greaser and Gergely, 1971[146]
Troponin III	Troponin II	Troponin T	Ebashi et al., 1971[145]
Component III	Component II	Component I	Murray and Kay, 1971[156]
Troponin C	Troponin I	Troponin T	Potter and Gergely, 1974[157]

from a common nomenclature that may outweigh the disadvantages of losing some of the symbols to which one has become attached personally. I would therefore like to propose that the following scheme be generally adopted: that the Ca-binding component of troponin (mol wt ~18,000) be called Tp C; that the inhibitory component (mol wt ~23,000) be called Tp I and that the tropomyosin-binding component (mol wt ~37,000) be called Tp T. [Today these are known as TnC, TnI and TnT].

There is general agreement that only Tp C binds significant amounts of calcium over the range of Ca^{++} concentrations in which regulation occurs (10^{-7}-10^{-5} M), and that Tp C on its own has no effect on the ATPase of actin plus myosin plus tropomyosin. Tp I acts as an inhibitor of actomyosin ATPase […] [and the] presence of Tp T is needed for the normal functions of the original troponin complex to be fully reconstituted, but there are still some residual divergences about the precise role of this component. However, there is general agreement that the troponin complex contains one unit of each of the three components mentioned, and that in the presence of one mole of tropomyosin, such a complex can regulate the activity of about seven actin monomers. (Hugh E. Huxley in 1972[136] with permission Cold Spring Harbor Laboratory Press)

The thin-filament "functional unit": (A_7TmTn)

The thin-filament consists of individualized functional units each with seven actin monomers spanned by one tropomyosin dimer and one troponin complex (A_7TmTn).

It is interesting to note (citations for the next statements can be found here[158]) that cTnI solely inhibits the interaction between myosin and actin in the presence of tropomyosin. In the absence of tropomyosin and cTnT, cTnC is insensitive to Ca^{2+}, that is, it binds to cTnI and removes its inhibitory function, irrespective of the absence of Ca^{2+}. Additionally, cTnI can maximally inhibit ATPase activity, in the absence of tropomyosin, if it is in a 1:1 ratio with actin. In the presence of tropomyosin, solely a single cTnI subnit per each four actin monomers is necessary to inhibit contraction,

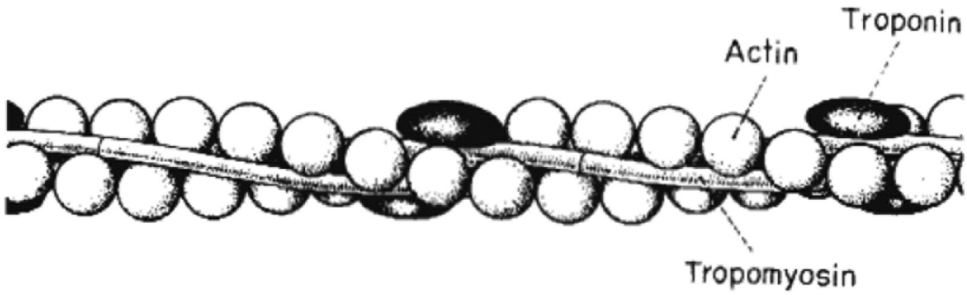

Figure 6. An early model of the thin-filament structure. (Figure adapted from Ebashi et al, 1969[101] with permission Cambridge University Press)

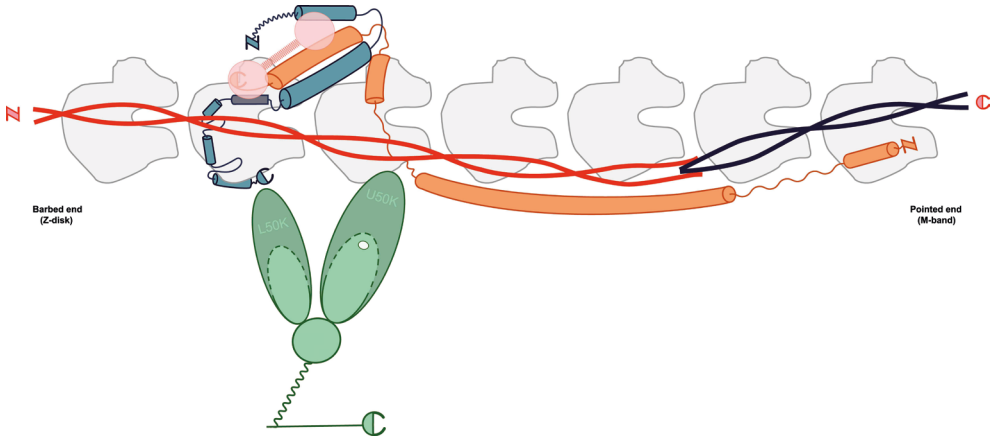

Figure 7. Schematic drawing of the thin-filament functional unit. Five actin monomers (gray) spanned by one tropomyosin dimer (red) and one troponin complex: cTnC (pink), cTnI (blue) and cTnT (orange). Letters "N" and "C" depict N- and C-terminal protein ends, respectively. Dark-blue tropomyosin depicts near-neighbor tropomyosin dimer interaction.[159,160] Myosin-S1 is depicted in solid green (light-green myosin-S1 to better understand its transition states). The orientation of thin-filament proteins is: the N-terminal region of cTnT points towards the pointed end (M-band), while the core domain of the troponin complex is oriented to the barbed end (Z-disk).[161] Interacting sites and structural regions of actin-tropomyosin-troponin proteins are matched in accordance with available literature[162]. Cardiac TnI residues 1-34 are arbitrarily positioned. Our figure follows the proposed mechanism for Ca^{2+}-regulation of contraction proposed by Murakami et al.[163] (Figure adapted from Sequeira et al, 2013[162] with permission Wolters Kluwer Health, Inc)

which supports for the existence of cooperativity, or cooperation, in muscle. The interaction of cTnC to cTnI is increased and strengthened by Ca^{2+}, in the presence of cTnT and tropomyosin.

The third filament: titin

The existence of another filament was initially observed by Hugh E. Huxley and Jean Hanson[70] in 1954, when through actin-myosin extractions the authors showed that the sarcomere remained intact. The authors[70] proposed that an elastic component called the "S-filament" provided continuity between an actin filament and the opposite actin filament in a sarcomere, and attached to myosin.

> *The backbone of the muscle fibril is made up of actin filaments which extend from the Z-line up to one side of the H-zone, where they are attached to an elastic component (not the series elastic component) which for convenience we will call the S-filaments. The S-filaments provide continuity between the set of actin filaments associated with one Z-line and that associated with the next. (Hugh E. Huxley and Jean Hanson[70] with permission Nature Publishing Group)*

Nonetheless, it was difficult at the time to confirm the existence of such an elastic and integrative component. Years later the existence of a giant elastic protein called "connectin" was reported consistent with the earlier proposed S-filaments.[164] Today it is better known as "titin"[165] based on its large proportions (molecular weight ranging from 3MD-3.8MDa[166,167]). Titin is the third-most abundant filament protein by weight.[168] Partially responsible for the generation of resting tension, titin is also the mechano sensor of the sarcomere.[169]

The N-terminal region of titin

This part of titin resides in the Z-disc and interacts with actin[170] and possibly with α-actinin[171,172] via 45 amino acid repeat regions (Z-repeats) that provide a mechanism of Z-disc assembly resulting from alternative splicing.[173] The I-band region of titin is the extensible region and consists of three elastic components that act as a spring element (Figure 8): 1) tandem immunoglobulin (Ig)-like domains with proximal

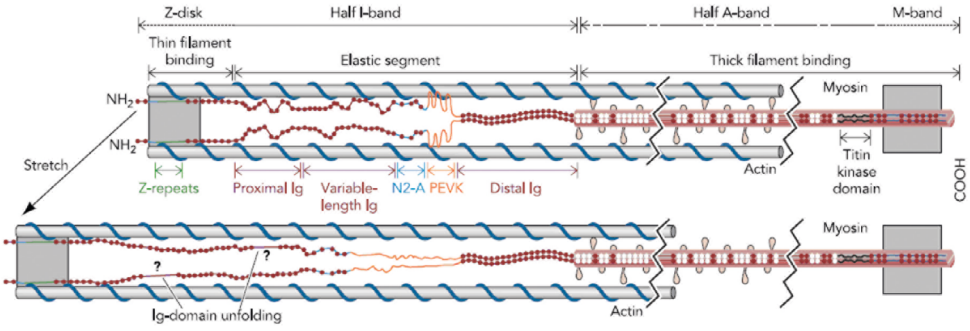

Figure 8. Schematic overview of titin depicted in half-sarcomere. Note the extension of the elastic components of titin when the sarcomere is stretched (Figure adapted from Linke W, 2010[169] permission not required)

(near Z-disc) and distal (near I-A regions) segments; 2) the PEVK sequence-region rich in proline (P), glutamic acid (E), valine (V) and lysine (K); and 3) the N2B and N2BA elements (both isoforms contain N2B segments, but only the N2BA has the N2A element).[167]

C-terminal region of titin

The C-terminal A-band region of titin is inextensible. It interacts with thick-filament and associated proteins, including myosin and cardiac myosin-binding protein C (cMyBP-C).[174-176] In the M-band, titins from opposing half-sarcomeres intersect and interconnect with M-band proteins, thereby forming a continuous filament from the M-band towards the Z-disc.[177,178] Titin may be arranged in the thick-filament as a dimer[179] and presumably a bundle of "*six titin molecules connect each end of the thick-filament to the Z-disk" (Ahmed Houmeida and colleagues[180] with permission Elsevier).*

Titin is present in both skeletal and cardiac muclse, but differs in its size.[181] Cardiac isoforms are smaller, ranging from 3MDa (N2B) up to over 3.2MDa (N2BA),[182] opposite to the even larger skeletal isoforms (N2A) that can reach as much as 3.8MDa.[167,169]

Titin and its "elastic" tension of muscle

Under resting conditions, striated muscle resists muscle lengthening by producing passive tension in response to stretch. In the heart, titin accounts for approximately 80% of total passive tension between physiological operating sarcomere lengths - 1.8 to 2.2 μm. When over-stretched (>2.2 μm), where the contribution of collagen is greater, the titin stretch-based passive tension remains high, indicative of the central role of titin to respond to stretch.[183,184] Passive (resting) tension results from the extensible I-band spring segment that elongates as sarcomere length increases. The tandem Ig-like segments are the first to extend, followed by the PEVK segment and lastly, the elongation of the N2B segment.[177,178] The flexibility and stretch-based passive tension of the titin spring elements can be regulated by two major mechanisms: a fast "acute" modulation by post-translational modifications via protein phosphorylation and a "chronic" isoform shift due to alternative splicing of the I-band encoding region of the titin (*TTN*) gene, giving rise to different isoforms.

MyBP-C, thick-filament associated component

The thick-filament that titin is not solely bound to myosin, but also to another protein in the C-zone of the A-band.[174,185,186] Called "C-protein"[187,188] or "cMyBPC"[189], this protein appears to hold a central role in cross-bridge binding[190,191] and cycling kinetics[191,192].

cMyBP-C mainly consists of immunoglobulin-like C2 domains (eight domains) and three/four fibronectin type-III domains. In addition, cMyBP-C has a proline-alanine

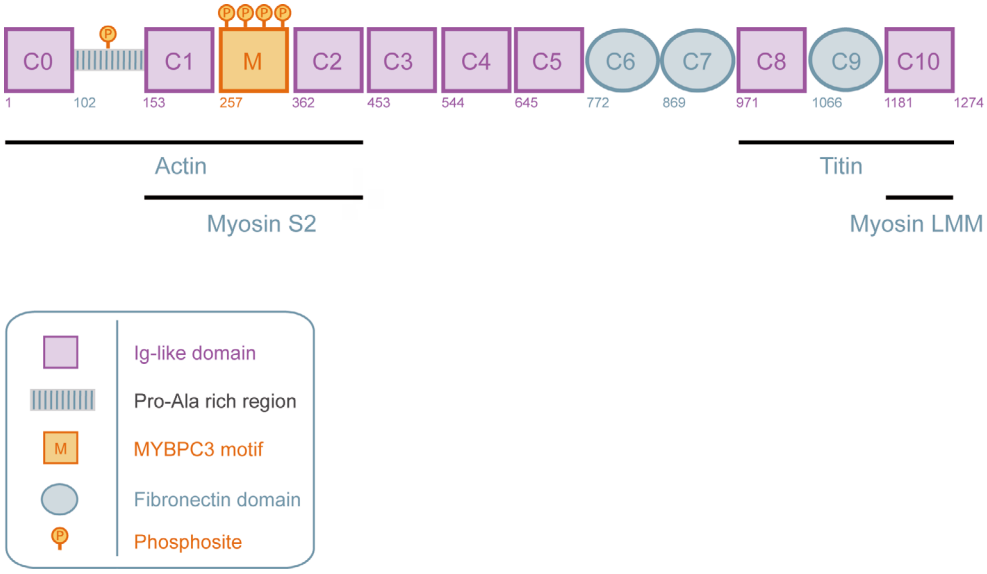

Figure 9: Schematic domain structure of cMyBP-C. Cardiac MyBP-C consists of eight Ig-like and three fibronectin domains labeled C0 (N-terminus) through C10 (C-terminus). Two additional domains are present in the N-terminal part of the protein, the Proline-Alanine rich region (PA) and the M-domain (M). Four phosphorylation sites, Ser275, Ser284, Ser304 and Ser311, have been described in the M-domain. A recent study[193] revealed a novel phosphorylation site on serine 133 in the PA region (Figure from Sequeira et al, 2013[194] with permission Springer).

rich region between domains C0 and C1 and a M-domain between domains C1 and C2 (Figure 9). Both linker domains have important functional roles - *vide infra*.

MyBP-C was first identified in 1973 by Offer et al.[187] from skeletal muscle. While MyBP-C's interaction with myosin[187] and actin[195-197] were reported soon after its discovery, the exact role of MyBP-C in muscle contraction remained poorly understood. Discovering the individual interaction partners of the N' and C' domains was key to understanding the function of MyBP-C in regulating contraction.

C-terminal region of MyBP-C

The C-terminal domains are important for the binding of MyBP-C to thick-filaments[198] that occur via interaction with the LMM region of myosin-heavy chain (MHC)[199,200]. Although the C10 domain was identified as the myosin LMM binding domain, domains C7-C9 are also needed for proper incorporation into the thick-filament.[198] This might be mediated by the interaction of the C' domains of cMyBP-C with titin, which involved domains C8-C10.[175]

N-terminal region of MyBP-C

While the C-terminus of cMyBP-C is important for its location and anchoring to the thick-filaments, the N-terminus is the region through which cMyBP-C exerts its regulatory role on contraction. The best understood aspect of cMyBP-C's function is its effect on cross-bridge cycling kinetics (Figure 10). This effect is mediated by the interaction of the cMyBP-C N-terminus with the MHC "neck" region.[201] This S2 region is the hinge region of MHC and connects the LMM to the myosin head (S1 domain). By binding to this region, cMyBP-C can slow cross-bridge cycling kinetics (Figure 10). The cMyBP-C/S2 interaction is phosphorylation-dependent and the phosphorylation sites in the M-linker domain are important for modulating this interaction.[202] When the cMyBP-C M-domain sites are not phosphorylated, cMyBP-C binds to S2. Upon phosphorylation of cMyBP-C, this interaction is lost.[202] Therefore, the cMyBP-C M-domain, together with the adjacent C1 and C2 domains, are thought to be the sites of interaction with myosin cross-bridges.[201,203] More controversial has been cMyBP-C's interaction with actin. Although cMyBP-C was reported to interact with actin *in vitro* soon after its discovery[196,197], the *in situ* visualization of cMyBP-C's direct interaction with actin in intact muscle was lacking. In recent years, accumulating evidence provided by *in situ* 3D reconstruction approaches (X-ray neutron scattering[204], negative EM staining[205,206] and electron tomography[207]) demonstrated that cMyBP-C interacts with actin. The N-terminus of MyBP-C projects towards the thin-filament making direct contact with actin. Phosphorylation of sites in the M-domain weaken its interaction with actin [208].

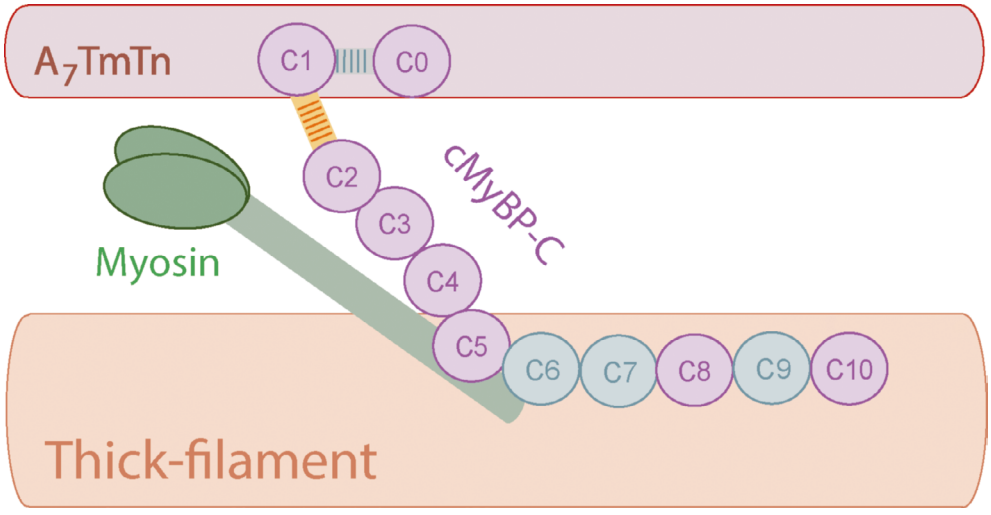

Figure 10. Schematic structure of cMyBP-C. cMyBP-C consists of eight Ig and three fibronectin domains labeled C0 (N-terminal) to C10 (C-terminal), with two additional linker domains the PA (Proline-Alanine; light blue stripes) region between C0 and C1, and the M-domain (M; yellow and orange sripes), between C1 and C2. The C5-C10 domains extend along the thick-filament, while the C0-C4 extend to the thin-filament. A_7TmTn depict a functional unit composed of 7 actin monomers, 1 tropomyosin (Tm) dimer and 1 troponin (Tn) complex.

In addition, recent evidence suggests that the N-terminal extension of cMyBP-C binds the low Ca^{2+}-state (blocked state, B-state; further discussed) position of tropomyosin on actin, indicating that cMyBP-C can interfere with tropomyosin-actin interactions and regulate thin-filament transitions [205]. The functional implications of the putative actin/cMyBP-C interaction are not yet fully understood. Another sarcomeric protein that was recently identified to interact with cMyBP-C's N-terminus (more specifically the C0 domain) is the regulatory light chain.[209] Again, the functional consequences of this interaction are not yet understood.

CENTRAL ROLE FOR Ca^{2+} IN MUSCLE CONTRACTION

It is well established that cardiac muscle contraction, which was defined as "excitation-contraction coupling" by Alexander Sandow in 1952[210], is initiated by electrical activation of cardiomyocytes, resulting in muscle contraction.

> *When a stimulus is applied to muscle, the response is indicated first by excitation which is set up in the membrane of each reacting fiber, and then by contraction which is a function of the substance within the membrane […] We shall designate the entire sequence of reactions–excitation, inward acting link, and activation of contraction by the term excitation-contraction (E-C) coupling. (Alexander Sandow[210] with permission Yale J Biol Med)*

Central to the excitation-contraction coupling is the increase in intracellular [Ca^{2+}] resulting after electrical activation of the membrane. The first indications for the importance of Ca^{2+} in the activation of cardiac muscle were demonstrated in the 1880s by Sydney Ringer[211,212], who observed that the ventricle of frog hearts beat faster when he used a solution prepared from tap water supplied by the New River Water Company in England, but not when distilled water was used. The difference was due to Ca^{2+} in the tap water. Ringer could maintain cardiac contractions if $CaCl_2$ and KCl were added during saline perfusion (0.75% NaCl) at concentrations of about 0.5 and 1.3 mM, respectively.

> *I find that calcium, in the form of lime water, or bicarbonate of lime or chloride of calcium, even in minute doses produces the changes in the ventricular beat described in my former paper. (Sydney Ringer[212] with permission John Wiley and Sons)*

The great next step for Ca^{2+} research was reported by Locke and Rosenheim in 1907[213] using Ringer's solution (0.75% NaCl, 0.5 mM $CaCl_2$, 1.3 mM KCl) who showed that,

> *If both calcium and potassium (ions) are omitted from the Ringer's solution, metabolic action in the heart still continues, although actual contractions are minimal or absent. […] Calcium is necessary for the conversion of the heart's chemical energy into the mechanical energy of its beat, while potassium is more necessary for the merely*

chemical processes of cardiac activity. (Locke and Rosenheim[213] with permission John Wiley and Sons)

Similar observations were made in 1913 by George R. Mines, who reported that a Ca^{2+}-free solution generates normal action potentials, but no mechanical response.[214] Further progress was made in 1940 by Heilbrunn[215] who showed that damaged muscle fibers could generate contractions in solutions containing high [$CaCl_2$] (>20 mM), and in 1942 Bailey[216] observed that mM concentrations of Ca^{2+} activated the ATPase activity of myosin. The following is an excerpt from Kenneth Bailey's paper[216], which summarizes the available evidence known at the time to support the Ca^{2+}-hypothesis:

The facts which seem to have some relevance when we attempt a correlation of our present data with the actual process of contraction can be summarized as follows: (1) Myosin and ATP-ase are identical or so closely associated that we can regard them as a single entity. (2) Myosin is the contractile element disposed in the form of folded chains lying roughly parallel to the axis of the fibre. (3) The reaction which is capable of supplying free energy for contraction and which is most closely associated in time with the act of contraction is the breakdown by ATP-ase of ATP to ADP and phosphate. (4) Of the divalent ions present in muscle, ATP-ase is specifically activated by the Ca ion. (5) Ca is necessary for muscular contraction. Its importance in maintaining the response of isolated muscle to stimulation has been known from the earliest systematic studies in muscle physiology. We may recall the classical experiments of Ringer and of Locke, and especially those of Mines [1913] who discovered that the excitatory process could proceed in the absence of Ca, although the muscle was unable to contract. This recalls the earlier experiments of Locke and Rosenheim [1907], who concluded that 'The State of the active surface in the absence of Ca is such that […] an increase of surface energy cannot take place'. (6) The electrical change accompanying excitation is connected with the diffusion of ions in the region of the excited area. Contraction is also accompanied by ionic changes, and although little is known of the divalent ions and much of the evidence is conflicting, there appears to be an increase of diffusible Ca [Weise, 1934]. The exchange of univalent ions during contraction, e.g. the loss of K in exchange for Na [Fenn and Cobb, 1936], is well established and would be expected to affect the distribution of divalent ions. (7) The Ca ion cannot be replaced by Mg, either for the maintenance of contraction or as activator to the ATP-ase system. The presence of Mg in muscle has been generally associated with other phosphorylating enzymes for which it is a specific activator. (Kenneth Bailey[216] permission not required)

The essential role of Ca^{2+} in muscle contraction and relaxation was still although obscure in the 1960s as noted by Ebashi and Endo[217]:

It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation. (Ebashi and Endo[217] with permission Elsevier)

In the 1950s it was well established that ATP was required for contraction in concert with Ca^{2+}. However, muscle physiologists did not understand how muscle relaxation proceeded. Marsh[218,219] proposed that a "relaxing factor" of small molecular size existed, responsible for the relaxation of muscle. It was believed myokinase was responsible for the "relaxing/Marsh factor".[220,221] In 1954 and 1955, Bozler[222] and Watanabe[223] reported that in the presence of ATP, a synthetic compound called "EDTA" mimicked the action of this "relaxing factor". As summarized below by Bozler[222], EDTA by Ca^{2+}-chelation was responsible for muscle relaxation.

> *Szent Györgyi (1-3) discovered that ATP produces a strong contraction in muscle fibers which have been preserved in glycerol. Later Bozler (4-7) found that such fibers, which originally are in rigor, can be transformed into a state closely resembling normal relaxation by physiological concentrations of ATP. The fibers then are very extensible and plastic, and give a strong contraction on addition of small amounts of $CaCl_2$. This state can be produced and maintained only in the presence of Mg and only in rather fresh fibers. Following up work by Marsh (8) on the syneresis of muscle homogenates, Bendall (9) has shown that the ability of briefly extracted fibers to relax is due to the presence of a non-dialyzable factor contained in muscle extract, which will be called relaxation factor ("Marsh factor"). […] In the present paper the clarification of the mechanism of action of these factors will be attempted, particularly that of the relaxation factor. It was found that EDTA closely imitates all the effects of this factor. The results indicate that relaxation is caused by the inactivation of bound Ca. Previous evidence that the relaxed state is due to the formation of an enzymatically inactive ATP-protein complex was confirmed. (Emil Bozler[222] permission not required)*

Weber recognized in 1959[224] that actomyosin preparations only hydrolyzed ATP if, μM of Ca^{2+} was present. Final confirmation for the role of Ca^{2+} in muscle activation came from Ebashi in a series of papers in the 1960s[130,131,225-228] demonstrating that indeed μM [Ca^{2+}] was required for the superprecipitation of the actomyosin complex and that the "relaxing factor" was in fact a vesicular factor capable of removing and storing Ca^{2+}, derived from an organelle with an extensive tubular network - the sarcoplasmic reticulum (SR).

> *Together with our findings with the vesicular relaxing factor, we would picture the following sequence of events. In the resting state, most of the Ca in muscle is concentrated in the endoplasmic reticulum, [,..] the vesicular relaxing factor. Excitation of the muscle membrane will be conducted into the interior of the cell causing the endoplasmic reticulum to release Ca-the trigger for contraction. When the excitation is completed, Ca will be again captured by the endoplasmic reticulum and relaxation will follow. (Setsuro Ebashi[228] with permission Physical Society Japan)*

Ford and Podolsky[229] suggested that Ca^{2+} release from the SR could be induced by external Ca^{2+}, which was confirmed by Endo et al.[230]. Despite the strong evidence for the role of Ca^{2+} in contraction-relaxation at the time, several weaknesses were

presented by Weber and Winicur[231], who observed that some preparations of synthetic actomyosin (a mixture of myosin and actin separately prepared) were less sensitive to Ca^{2+}.

> *Some actomyosin preparations superprecipitate very little or not at all and hydrolyse adenosine triphosphate at one quarter or one-half of the maximal rate obtained on addition of $CaCl_2$ to give a concentration of 0.1 mM (Weber and Winicur[231] with permission American Society for Biochemistry and Molecular Biology)*

As shown previously, Ebashi reported that this was due to a third muscle component, tropomyosin.[130]

Roles of Ca^{2+} and ATP in muscle contraction

Cardiac muscle contraction is initiated upon electrical activation of cardiomyocytes and the resulting increase in intracellular [Ca^{2+}] and regeneration of ATP. In the 1970s the idea was raised that cross-bridge cycling occurs in two stages. First, the absence of Ca^{2+}, tropomyosin physically blocks the myosin-bindings sites on actin (the steric blocking model) and second, the concept that with raised intracellular [Ca^{2+}] myosin binds and induce force development.[232,233] McKillop and Geeves[65] in 1993 advanced the later ideas and proposed a three-state model of the thin-filament, in which myosin-binding to actin in the presence of Ca^{2+} does not occur in a single step, but in two steps that reflect changes in the affinity of myosin for actin.

> *We propose a three-state model of the thin filament. A 'blocked state' which is unable to bind S1 [myosin], a 'closed state' which can only bind S1 relatively weakly and an 'open state' in which the S1 can both bind and undergo an isomerization to a more strongly bound rigor-like conformation. (McKillop and Geeves[65] with permission Elsevier)*

In concert, Ca^{2+} and ATP characterize three distinctive states of a muscle fiber: i) relaxed, ii) activated and iii) rigor. In addition, a three-state model of thin-filament regulation comprises three distinct biochemical states of muscle: "blocked-state", "closed-state" and "open-state". Electron microscopy reconstructions[66] of thin-filament proteins confirmed the solution studies from McKillop and Geeves[65], and proposed new generic terms to "*avoid nomenclature with unintended connotations*"[66]: the blocked-state corresponds to the "blocked (B-state)"; the closed-state to "Ca^{2+}-induced (C-state)", and the open-state to "myosin-induced (M-state)" (Figure 11).[66]

Relaxed state, B-state

I. In the relaxed state where Ca^{2+} levels are low and ATP is present, the muscle does not develop active force and muscle stiffness is low. Corresponding to the physiological relaxation (diastole), it determines the amount of passive resting tension. At

the molecular level, the decline of Ca^{2+} levels is associated with uncoupling of Ca^{2+} from TnC, and relaxation proceeds.[64,69,234] In the B-state, tropomyosin sterically blocks myosin-binding sites on the outer domain of actin, thereby promoting inhibition of myosin S1 binding to actin (Figure 11A). We know that specific regions of TnI bind to the outer domain of actin and thereby "drag" tropomyosin with it, pulling it away from the inner groove of actin, blocking actomyosin interactions in the outer region.[163,235]

Activated state

II. In an activated state, where Ca^{2+} levels are high and ATP is hydrolysed, the muscle generates active force, shortens and becomes very stiff. It corresponds to the physiological contraction phase - systole. As cytosolic free Ca^{2+} increases, it binds to regulatory sites on TnC, resulting in the structural arrangement of the TnC subunit.[236] Because TnC is structurally attached to TnI[237,238], it moves TnI away from actin, shifting tropomyosin back towards the inner domain of actin. The uncovering of the outer domain is propagated from one actin to, at least, fourteen neighboring actins (two functional units), leading to cooperative activation of the thin-filament.[239-241] Activation takes place in two distinct biochemical steps:

C-state

II.1. A movement of ~25° of tropomyosin around actin, corresponding to the C-state, exposes most of the myosin-binding sites (Figure 11B).[135,139] Nonetheless, the myofilament is not yet activated because non-tension-generating cross-bridges bind weakly to actin (weakly-bound cross-bridges, myosin-ADP-P_i). Notably, not only does tropomyosin change its orientation, but slight movements of actin subdomains have also been reported.[242,243] McKillop and Geeves[65] reported that this specific transition represents the state of weak binding of the S1 (acto-S1-ATP or acto-S1-ADP-P_i) to actin.[244,245] Weak-binding cross-bridges are defined by low affinity to actin, very fast myosin attachment to and detachment from actin (faster attachment/detachment kinetics), and inability to activate thin-filament regulatory units (A_7TmTn).

M-state

II.2). The third and last state, the M-state, involves the release of P_i from the cross-bridge and strong-binding cross-bridge formation (myosin-ADP) that induces an extra ~10° movement of tropomyosin on the actin filament, resulting in myofilament contraction and force development (Figure 11C).[66,139] The shape complementary of tropomyosin to actin (Gestalt binding[246]) might prevent tropomyosin from extensively rolling away from actin.[247] Strong-binding cross-bridges are defined by higher affinity to actin, myosin binds very strongly to actin and detaches very slowly (slower attachment/detachment kinetics), and the ability to activate thin-filament regulatory units.

Overall, the troponin complex inhibits a kinetic transition by trapping the position of tropomyosin.[123] An increase in the free acto-S1-ADP concentration, enhanced

by cooperative thin-filament activation,[244,248,249] in a Ca^{2+}-dependent manner, is the trigger for S1-strong-binding and consequent cycling and contraction.[65,241] A new cycle of relaxation ensues, if the Ca^{2+} levels decline and ATP is readily accessible.

Rigor state

III. In particular cases the rigor state can be induced (<0.1 mM ATP)[125], and although it is as stiff as the activated state,[250] the muscle does not shorten.[118,127]

MUSCLE MECHANICS: LENGTH-DEPENDENT CHANGES

Changes in length and load allow the heart to change its ventricular filling and regulate ventricular contraction and ejection. Ventricular filling fixes the initial sarcomere length-tension and optimizes the i) amount of force development, ii) the rate of force development and iii) the velocity of shortening against afterloads, i.e. cardiomyocyte workput, ultimately regulating ventricular contraction and ejection.[261] The Frank-Starling relation thus allows the heart to work on a beat-to-beat basis, capable of adjusting the output of both its sides to any alteration affecting venous return, or preload. The sarcomere length-tension relationship in skeletal and cardiac muscle differs, though active tension shows similar dependence on length. The changes in skeletal muscle are discussed below and enable the understanding of the Frank-Starling relation.

Skeletal muscle length-dependency

Blix[16] and von Kries[17,18] proposed that the energy of a contracting muscle is a function of its length and that muscle shortening depends on its load. Evans and Hill[262] in the 1910s showed that developed tension grew by increasing fiber length. In addition, a parallel increase of heat production was observed.

> *The experiments of which the results are described here were undertaken at the suggestion of Professor Starling, with the view to testing the theory put forward by Blix that the variable factor among the mechanical conditions which determines the mechanical performance and the heat production of muscle is the initial length of the muscle and to find what relation, if any, exists between the length of the muscle and the tension and heat which it produces on isometric contraction. (Evans and Hill[262] with permission John Wiley and Sons)*

The complete length-tension relation for isolated skeletal muscle fibers was for the first time provided by Ramsey and Street[263] in 1940 (previous experiments rarely exceeded 30% resting length changes), who demonstrated the biphasic shape alteration of tension development upon length. Almost three decades were needed to combine the results obtained from Ramsey and Street[263] to the growing advances obtained from the analysis of the ultrastructure of skeletal muscle fibers - sliding filament theory. In an historic paper published in 1966, Gordon, Andrew F. Huxley

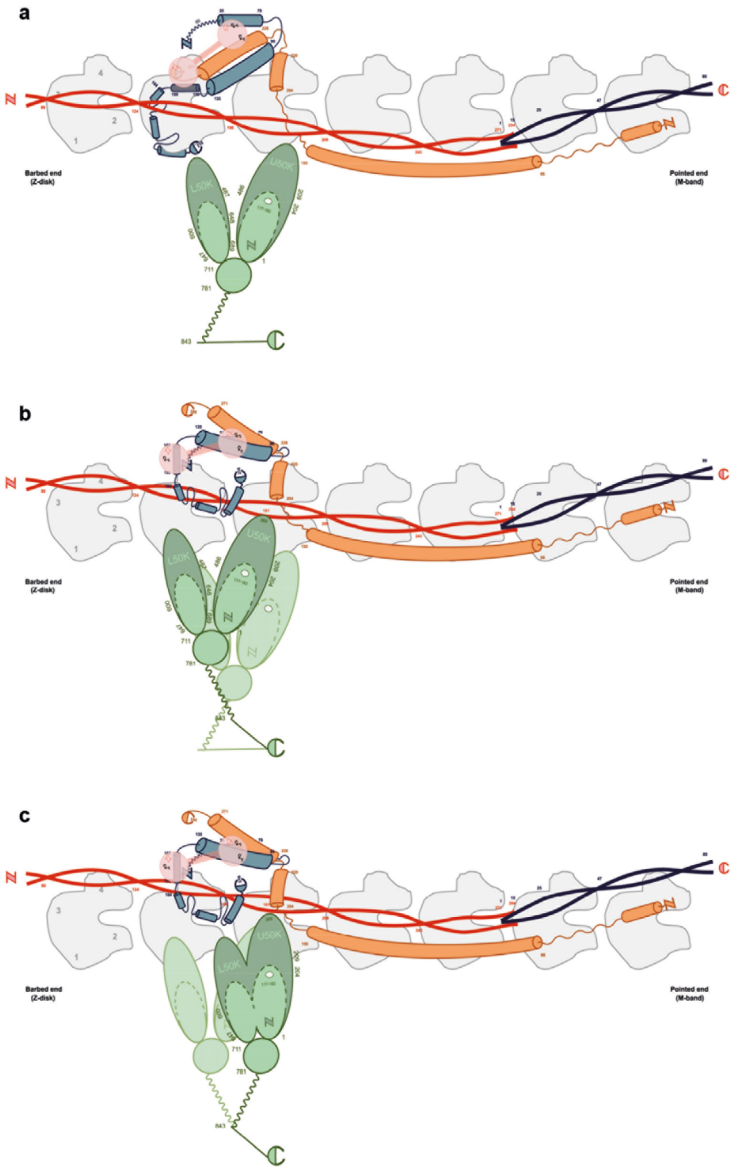

Figure 11. Schematic drawing of the thin-filament functional unit. Seven actin monomers (gray) spanned by one tropomyosin dimer (red) and one troponin complex: cardiac troponin C (pink), cardiac troponin I (blue) and cardiac troponin T (orange). "N" and "C" indicate the N- and C-terminal ends of proteins. This representative diagram is based on the structure of actin subdomains,[251,252] the position of tropomyosin on F-actin[66,135,139] and the core domain of human troponin.[237,253] The tropomyosin overlap region (head-to-tail) depicts interaction with near-neighbor tropomyosin dimer (dark-blue).[159,160] The orientation of thin-filament proteins is as follows: the N-terminal region of cardiac troponin T points towards the pointed end (M-band), while the core domain of the troponin complex is oriented to the barbed end (Z-disk).[161] Interacting sites and structural location of actin-tropomyosin-troponin proteins were matched the best as possible in accordance with the available

literature.[160,237,254-260] A. B-state (blocked); when ATP is present and cytoplasmic [Ca^{2+}] is low and is not bound to cTnC, tropomyosin is sterically blocking the myosin-binding sites on actin. B. C-state (Ca^{2+}-induced); cytoplasmic [Ca^{2+}] rises such that Ca^{2+} binds to cTnC, inducing conformational changes of the troponin complex, resulting in a ~25° movement of tropomyosin on the thin-filament, thereby exposing most of the myosin-binding sites on actin. Note the movement of tropomyosin away from subdomains 1 and 2 of actin. In the C-state the myofilament is not yet activated as non-tension-generating cross-bridges bind weakly to actin. C. M-state (Myosin-induced); the strong-binding of tension-generating cross-bridges induces a ~10° movement of tropomyosin on actin, resulting in myofilament activation and contraction. Note the transition of tropomyosin into subdomains 3 and 4 of actin (Figure adapted from Sequeira et al, 2013[162] with permission Wolters Kluwer Health, Inc).

and Julian[264] confirmed the biphasic shape of tension upon length alterations and suggested that such changes directly resulted from the overlap of individual cross-bridges. They demonstrated that active tension in skeletal frog muscles consisted of a maximal tension plateau region between sarcomere lengths of 2.0 and 2.25 μm; a region wherein myofilament overlap of thick- and thin-filaments was optimal and constant (Figure 12). Stretching the muscle above 2.25 μm progressively reduced active tension (known as the descending limb of the active tension-length curve) and reached a zero level at 3.65 μm sarcomere length, where myofilament overlap ceased.[264] Upon shortening the muscle below 2.0 μm (known as the ascending limb of the active tension-length curve) active tension decreased, which was proposed to be the result of thin-filaments colliding at the center of the sarcomere (double overlap) in addition to collision of the thick-filaments at the Z-disc. However, a poor correlation between the ascending limb and the number of active cross-bridges was subsequently suggested, so that as the muscle is shortened below slack length the number of cross-bridges remains constant while tension drops.[265-268] These observations provided the first indications that factors other than the degree of myofilament overlap of thin- and thick-filaments existed, and accounted for deactivation upon shortening. In agreement, Taylor and Rüdel[269,270] demonstrated that the addition of low caffeine concentrations (which causes the release of Ca^{2+} from the SR) to the bathing solution increased the developed tension at short sarcomere lengths. A similar study was performed by Close[271] in frog skeletal muscles, showing that inducing twitch- or tetanic-activation generated different length-tension curves. Close proposed that length-independent changes in activation could also play a role to increase tension.[271]

> *It may be concluded from this that the principal variations in the length dependence of the twitch are the result of differences in some extrinsic process involved in activation and not differences in the intrinsic strength of the contractile material. (Close R.I.[271] with permission John Wiley and Sons)*

FRANK-STARLING RELATION

In 1895, using the frog heart, Otto Frank[11] measured isovolumic pressures developed at varying lengths. Extending the findings of Blix and von Kries, Frank[11,19] observed

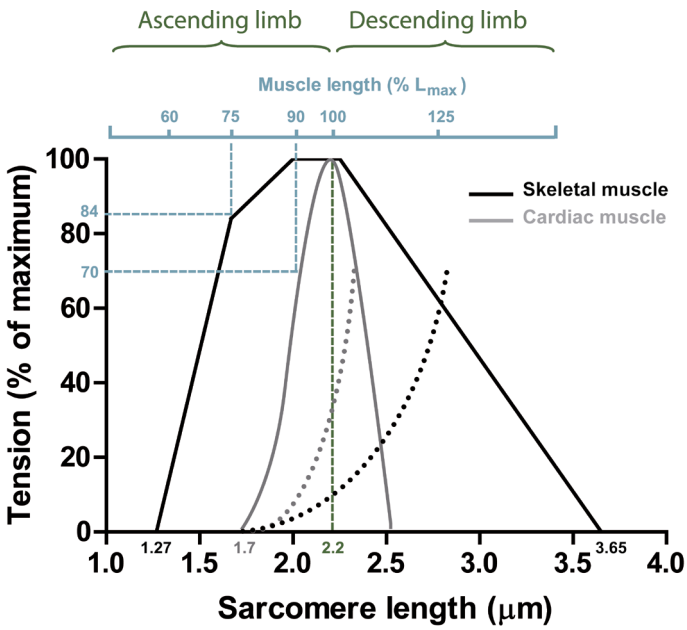

Figure 12. A comparison of length-tension relations for skeletal and cardiac muscle. Skeletal muscle. As proposed by Gordon et al.[264] using the frog heart, a maximal plateau region over the range of 2.0 and 2.25 μm sarcomere length is expected due to optimal and constant myofilament overlap. When the muscle is stretched above 2.25 μm (descending limb) active tension declines to almost zero at a sarcomere length of 3.65 μm. At shorter sarcomere lengths (below 2.0 μm) (ascending limb) the thin-filaments collide in the middle of the sarcomere, and thick-filaments collide at the Z-disc and tension ceases. Cardiac muscle. The myofilament overlap theory that was the basis for skeletal muscle length-tension relations cannot account for the cardiac length-tension relationship. Apart from the smaller sarcomere lengths at which the mammalian heart operates (estimated physiological levels range from 1.8 to 2.2 μm), cardiac muscle was demonstrated to present length-dependent changes in activation. Please note that skeletal muscle almost fully activates at 75% L_{max} (length at which force is maximal), which contrasts with cardiac muscle where at the same % of Lmax, active tension is zero. Lengthening cardiac muscle an extra 15% in length (~90% L_{max}) raises the developed tension from 0 up to 70%, hence active tension in cardiac muscle is length-dependent. Diagrams adapted from Gordon and Julian[264], Sonnenblick and Skelton[272], and Allen and colleagues[273].

that the developed pressure was directly proportional to the initial (diastolic) tension. Nonetheless, Frank's experiments to determine whether an increase in the force of contraction was either related to the initial **tension** or **length** of the muscle fibers were inconclusive.[20] Starling and colleagues[7,20,22] measured cardiac shortening as a function of cardiac output and its intrinsic relation to initial EDV, and demonstrated that the initial fiber length, rather than tension, is the main determinant for contraction.

The performance of skeletal muscle length-tension relationships were investigated with cardiac muscle by Abbott and Mommaerts[274] and later reexamined in more detail by the groups of Sonnenblick[275-277] and Grimm[278,279]. Both groups demonstrated that active tension exhibited a similar dependence on length to skeletal muscle, namely

both change with sarcomere length. However the shape of cardiac length-tension curves is distinctly different. Skeletal muscle is almost fully activate at 75% of its optimal length (L_{max}; muscle length at which tension is maximal) compared with cardiac muscle where active tension is zero at the same % of L_{max} (Figure 12).[272,273] Stretching cardiac muscle an extra 15% (~90% L_{max}) increases developed tension from 0% up to 70% (Figure 12).[272,273] Based on this observation, it was suggested that active tension in cardiac muscle is length-dependent.

Several other inconsistencies between skeletal and cardiac muscle were reported, such that maximal tension in the heart muscle only presents a peak at maximal lengthening in contrast to the plateau in skeletal muscle, and that the decline in active tension (both ascending and descending limb) is much steeper for cardiac. A simple approach based on myofilament overlap could not account for the observed differences between skeletal and cardiac muscle.[272,276]

With the growing evidence from skeletal muscle for the role of Ca^{2+} in the "manipulation" of length-tension curves, it was apparent that factors other than the degree of myofilament overlap could explain the striking differences in length-tension curves and the enigmatic length-dependence of heart muscle.

Please note that later findings in the 1980s from Edman and colleagues[280] showed that the length-tension curve in skeletal muscle exhibits a much smoother shape than proposed by Gordon, Andrew F. Huxley and Julian[264]. Specifically, the length-tension relation does not have a pronounce plateau region at 2.0- 2.2 µm sarcomere length, while the descending limb was also non-linear. Instead there was a slight sigmoid shape[280], where the extrapolated zero tension level was reached at 3.49 µm rather than 3.65 µm sarcomere length.

Length-dependent activation: Ca^{2+} as a modulator

It was previously demonstrated in membrane-permeabilized skeletal muscle that the shape of the length-tension curve could be varied by adding µM Ca^{2+}.[281-283] To this extent, Fabiato and Fabiato[284] initially suggested that shortening the cardiac muscle partially inhibits the Ca^{2+}-triggered Ca^{2+}-release from the SR by monitoring free Ca^{2+} with the Ca^{2+}-sensitive photoprotein "aequorin". The authors however later dismissed their claims and provided proof that the contractile machinery itself is Ca^{2+}-sensitive to alterations in muscle length at sarcomere lengths greater than 2.35 µm (descending limb).[285] Later evidence demonstrated that indeed increasing sarcomere length (over the entire sarcomere length range) increases myofilament sensitivity to Ca^{2+}, thus "cementing" the myofilament length-dependent activation hypothesis.[286,287]

CURRENT CONCEPTIONS OF THE MECHANISMS UNDERLYING LENGTH-DEPENDENT ACTIVATION

Over a century of research on the Frank-Starling effect has elucidated our understanding of the fundamental mechanical basis of muscle contraction. The Frank-

Starling relation dictates that an increase in fiber length enhances the maximal force generating capacity and Ca^{2+}-sensitivity of myofilaments, leading to increased force development.[261,288]

Although a unifying concept that explains how myofilaments "sense" length alterations is still to be proven, stretch-induced effects rather than changes in filament spacing, dominate the literature. Also, changes in Ca^{2+}-activation upon muscle lengthening have to be accounted for. Overall, myofilament length-dependent activation is the composite of several synergistic mechanisms. Length-dependent activation is associated with: 1) increased Ca^{2+}-affinity of cTnC; 2) alterations in interfilament lattice spacing; 3) titin-induced stretch and formation of strong-binding cross-bridges; 4) cTn complex changes; and 5) cooperative mechanisms.

1. cTnC-dependent alterations

1.1. Ca^{2+}-affinity to cTnC

Two hypotheses were proposed to explain length-dependent activation of the contractile apparatus by Ca^{2+}. The first is the length-dependent regulation of Ca^{2+} release by the SR suggested by Fabiato and Fabiato[284], but this has not been confirmed by others. The second hypothesis, that length-dependent modulation of Ca^{2+}-affinity by cTnC is emerging as a solid candidate.[289-293] Allen and Kurihara[289] microinjected aequorin in isolated papillary and trabeculae muscles, and observed a rise in intracellular [Ca^{2+}] following a quick step release during contraction, which they attributed to dissociation of Ca^{2+} from the contractile proteins. Housmans et al.[290] observed similar phenomena. This view was further supported in membrane-permeabilized muscle preparations by Allen and Kentish[291] who concluded that Ca^{2+} was released by the contractile apparatus. Additional evidence by Hofmann and Fuchs[292,293] showed that length-dependent changes affect the Ca^{2+}-affinity of cTnC where a decrease in sarcomere length reduced the Ca^{2+}-binding affinity of cTnC.

1.2. cTnC is not a length-sensing molecule

It remains to be seen how cTnC "senses" sarcomere length alterations. Initially cTnC was considered to act as a "length sensor" itself. Babu et al.[294] reported that length-dependence of Ca^{2+}-sensitivity was substantially diminished when cTnC was exchanged by slow skeletal troponin C (ssTnC) in membrane-permeabilized cardiac muscle. They[294] attributed the isoform difference of cTnC as the basis for cardiac length-dependent activation compared to skeletal muscle. However this proposal was dismissed because other groups showed that length-dependent activation is independent of cTnC isoform differences.[295-297]

2. Interfilament lattice spacing vs. myocyte lengthening

It has been suggested that the increased Ca^{2+}-sensitivity upon myocyte lengthening results from lattice spacing reduction, which increases the proximity of myosin towards

actin. Alterations in interfilament lattice spacing, such as increases in the lattice spacing as a result of shortening, leads to sarcomere thickening and intracellular volume redistribution.[298-300] In turn, the developed active tension decreases via decreased approximation of myosin and actin filaments, and thus less strong binding cross-bridges are formed.[301,302] Although myocyte lengthening and subsequent lattice reduction are intimately linked, accumulating evidence suggests that lattice reduction itself does not play *per se* a major role in length-dependent cardiac muscle activation.

In 1977, Godt and Maughan[303] were able to vary the maximum tension of Ca^{2+}-activated fibers using high-molecular-weight polymers, such as dextran. Because of its inability to diffuse into the lattice spacing, dextran could compress the sarcomere and decrease the interfilament distance.[303] Next, it was found that variations in fiber width (as a function of lattice spacing) could be regulated by varying concentrations of dextran.[304] The groups of Moss[305] and Stienen[306] indicated that lattice spacing, rather than length, was responsible for the changes in Ca^{2+}-sensitivity in membrane-permeabilized skeletal muscles. These results were confirmed in skeletal[307-309] and cardiac[308,310] muscle preparations which demonstrated that osmotic compression (as a function of muscle width) enhances Ca^{2+}-sensitivity. These findings were in agreement with the proposition of Hofmann and Fuchs[292,311] who demonstrated that Ca^{2+}-binding to cTnC directly regulates cross-bridges interactions (rather than sarcomere length). Fuchs and Wang[312] observed that both Ca^{2+}-sensitivity and Ca^{2+}-affinity to cTnC were directly correlated with the lattice spacing but not with sarcomere length in cardiac muscle. This hypothesis, however, remains largely disputed and evidence from the last decade suggests another view. In the previous studies, alterations in lattice spacing as a function of dextran addition were not directly measured, but were indirectly based on alterations in muscle width. To visualize the interfilament lattice spacing as a function of dextran application, the group of de Tombe[313-315] used synchrotron X-ray diffraction in membrane-permeabilized and intact cardiac tissue. The authors observed that Ca^{2+}-sensitivity was not in a linear relation with interfilament spacing as a result of osmotic compression. Rather, compression of the lattice spacing with 4% dextran to match the decreased lattice spacing as observed when the sarcomere length is increased to the optimal length (≈2.2 μm) did not affect myofilament Ca^{2+}-sensitivity.[314,315] These studies are a major obstacle against the suggestion that interfilament lattice spacing is the major mechanism in the regulation of length-dependent activation.

3. Titin

Evidence from the last decade revealed that the giant elastic protein, titin, may be involved in the modulation of active tension and serve as a length-dependent sensor. Titin could exert such length-dependent behaviour by two possible mechanisms:

Titin-induced reduction of lattice spacing

A titin-based *passive* tension potentiation of cross-bridge formation could reduce the filament lattice spacing upon stretch.[316-318] Along these lines, a correlation between

enhanced length-dependent activation and higher levels of passive tension has been reported.[316,317,319] A recent study reporting a reduced myofilament force development and impaired length-dependent activation[320] in a rat with a homozygous autosomal mutation expressing a giant titin isoform (N2BA-G, ~3.9 MDa)[321] appears to support this idea.

Stretch potentiates titin-induced strong-binding cross-bridge recruitment

Another view, however, suggests that titin-induced stretch effects a reduction in lattice spacing. Titin is obliquely to the sarcomere axis and since it is attached to both myosin and cMyBP-C,[174-176] it may impose a passive strain on the thick-filament proteins, thereby reducing lattice spacing and changing the geometry of the cross-bridges.[318,322] Recruitment of strong-binding cross-bridges via a titin-induced stretch is vital to length-dependent and stretch-activation mechanisms. It has been reported in frog skeletal muscle that sarcomere lengthening increases myosin periodicity[323], such that the transition of the population of rested (order; on the axis plane of the thick-filament) cross-bridges to weak binding cross-bridges increases (disorder, Figure 13).[324,325] A similar finding was recently observed in cardiac muscle where the orientation of myosin heads becomes more perpendicular to the thick-filament axis when sarcomere length is increased.[326] Altogether these studies suggest that stretch-induced activation by titin-induced radial strain of the thick-filament is likely to increase the number of cross-bridges at the thick-filament, which would allow more myosins to attach to actin, in strong-binding states (Figure 13).

4. Cardiac troponin and thin-filament transitions: regulator of length-dependent activation

4.1. Stabilization of the B-state formation: length-dependent sensitive step

There is evidence to suggest that length-dependent activation is regulated via an "on-off" switch of the thin-filament.[328] The relevance of the transition from the B-state to the C-state for proper length-dependent activation has been shown by Smith and Fuchs[328] who were the first to provide evidence for a length-sensitive step in the transitions of thin-filament activation. A reduction in ionic strength (<0.05 M), known to shift the B-state equilibrium towards a stable C-state (where the disordered population of cross-bridges is increased[329,330]; i.e. it mimics the effects of stretch), coincided with impaired length-dependent activation.[328] Terui and colleagues[331] recently demonstrated that length-dependent activation is associated with titin-induced strain on the thick-filament, which is highly dependent on the troponin complex. The authors observed that reconstitution of cardiac thin-filaments with fast sTn reduced length-dependent activation to a level similar to that of skeletal muscle. In turn, reconstitution with cTn restored length-dependent activation and decreased Ca^{2+}-sensitivity. The authors associated the latter findings as an increased transition of the B-state towards the C-state.[331]

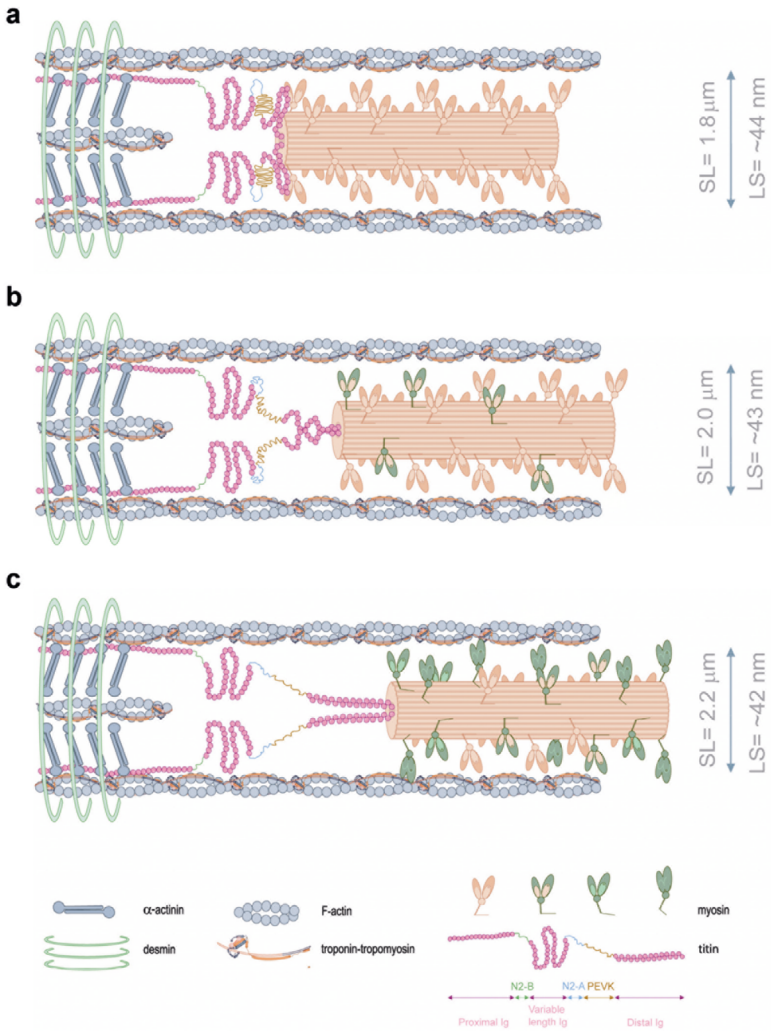

Figure 13. Schematic drawing of half-sarcomere at varying sarcomere lengths. Lattice spacing dimensions at each varying length were taken from Konhilas et al.[327]. As the muscle is stretched from a relatively short sarcomere length (A) to higher sarcomere lengths (B and C), lattice spacing becomes smaller with increased transition of order cross-bridges (A; projection of cross-bridges in X-ray diffraction studies) into disorder (active) states (B and C). The I-band region of titin is the extensible region and consists of three elastic components that act as a spring element: 1) tandem immunoglobulin (Ig)-like domain regions, with proximal (near Z-disc) and distal (near I-A regions) segments; 2) the PEVK sequence-region rich in proline (P), glutamic acid (E), valine (V) and lysine (K); and 3) the N2B and N2BA elements (both isoforms contain N2B segments, but only the N2BA isoform contains an additional N2A element).[167] Titin-induced stretch imposes a passive strain on the thick-filament proteins, reduces lattice spacing and changes the arrangement of cross-bridges. Distinct myosin colours are depicted to better illustrate the transition of ordered to disordered projections. α-actinin and desmin illustrate the Z-disc border. According to detailed calculations from Gordon and colleagues[128] ~1 cross-bridge binds each A_7TmTn. Note: cardiac myosin-binding protein C (cMyBP-C) was omitted to simplify the drawing and the width and sarcomere length dimensions are not to scale.

Recent findings from stretch-activation studies in insect flight muscle support the view that troponin-tropomyosin thin-filament transitions are central to the length-dependent response. Flight muscles require stretch-activation mechanisms in addition to Ca^{2+} to activate the muscle.[332,333] Because transition of the B-state requires Ca^{2+} to move tropomyosin to the C-state, insect flight muscles are switched "on" due to Ca^{2+}-induced binding to TnC; however, Ca^{2+} alone is not sufficient to uncover the myosin-binding sites - stretch is required. Since muscle stretching appears to increase myosin periodicity with orientation of myosin heads on the thick-filament[323,326], one can speculate that insect flight muscle requires the formation of strong-binding cross-bridges presumably by stretch in order to move tropomyosin and uncover myosin-binding sites. In support, Perz-Edwards and colleagues[334] recently demonstrated that stretch-activation of insect flight muscle requires the steric blocking-unblocking model (thin-filament transitions) for the regulation of the actomyosin complex and that stretching the muscle causes tropomyosin movement and uncovers myosin-binding sites on actin. The similarities between insect flight and vertebrate muscle thus suggest that passive strain imposed to the thick-filament increases the "activation" of cross-bridges which presumably via binding to troponin ("troponin bridges"[334]) and/or tropomyosin[247] regulate length-dependent activation. Because mutations on sarcomeric proteins can potentially impair the equilibrium affinity and thin-filament transitions, in Chapter 3 we conducted a large study in human HCM samples as to assess whether length-dependent activation is preserved in tissue with diverse sarcomeric mutations which cause cardiomyopathy. Indeed, we provide evidence that a reduced length-dependent increase in Ca^{2+}-sensitivity is a common mechanism underlying cardiac dysfunction in HCM with missense mutations, indicative for the important role of thin-filament transitions for myofilament length-dependent activation.

4.2. Post-translational modulation by protein kinase A (PKA) in length-dependent activation

The suggestion that PKA-mediated myofilament protein phosphorylation has a modulatory role in length-dependent activation comes from studies on ferret papillary muscles,[335] in which isoprenaline, a stimulator of the β-adrenergic receptor pathway, enhanced the length-dependent change in the force-Ca^{2+} relation. Reconstitution of cardiac thin-filaments with ssTnI showed higher myofilament Ca^{2+}-sensitivity, but significantly reduced length-dependent activation,[315,336] indicating a role for cTnI phosphorylation in length-dependent activation. In support, recent data from our group[337] and others[338,339] clearly demonstrates that PKA-induced phosphorylation of cTnI-Ser23/Ser24 is essential for length-dependent activation. One can speculate that because PKA-induced phosphorylation decreases cTnC-cTnI interactions, this leads to greater cTnI-tropomyosin interactions and hence, the B-state is favored; this is associated with less myosin-binding sites available on actin. In Chapter 6 we provide evidence that indeed the B-state is strengthened upon PKA-induced phosphorylation

which is partly the resulting contribution of cTnI but also of cMyBP-C. Finally, a study by Cazorla et al.[340] using transgenic mice lacking cMyBP-C demonstrated high Ca^{2+}-sensitivity and reduced length-dependent activation compared to wild-type mice, and that this could not be restored by exogenous PKA treatment. This study suggests that cMyBP-C is required for proper length-dependent sarcomere activation as well.

5. Cooperativity

5.1. What is cooperativity?

The sigmoidal relationship between [Ca^{2+}] and force and/or ATPase activity is one of the earlier demonstrations that the binding of Ca^{2+} appeared to activate muscle contraction in a cooperative fashion. Filo and colleagues in 1965[341] were the first to correlate tension as a function of the free [Ca^{2+}] using glycerinated skeletal and smooth muscle preparations. This observation was confirmed by Hellam and Podolsky[281] in membrane-permeabilized muscle preparations. The authors were the first to describe the relationship between free [Ca^{2+}] (defined by its inverse logarithm: pCa, i.e., $-\log_{10}$ of the [Ca^{2+}]) and force as a sigmoidal curve. Six years later, Donaldson and Kerrick[342] introduced the widely known term "pCa_{50}". When studying the effects of Mg^{2+} on muscle Ca^{2+}-contraction in membrane-permeabilized fibers, the authors observed no difference in maximal tension generated whether Mg^{2+} was present or not. However, to their surprise, if a submaximal tension comparison was made (at 50% of the maximum tension), Mg^{2+} was shown to reduce force generation. pCa_{50} is a widely used parameter to characterize the "Ca^{2+}-sensitivity" of a muscle system. In addition, Donaldson and Kerrick[342] also assumed the existence of a cooperative system:

> *The tension in the curves [...] rises from 10 to 90% of maximum in less than 2 pCa [...] units which is indicative of interacting sites. Because of the evidence for interacting sites these data were analyzed using the Hill equation (Hill, 1913[343]) which accounts for cooperative forces in the binding of a ligand to a macromolecule (Hill, 1913[343]; Loftfield and Eigner, 1969[344]). (Donaldson and Kerrick[342] permission not required)*

The coefficient of Hill (or nH) is thus an indication of the relative number of interacting sites and represents a measure of the cooperativity of Ca^{2+} activation of the contractile machinery. A hypothetical system with a nH value of 1 describes a one-to-one relation, where 1 mole of Ca^{2+} activates 1 functional unit (A_7TmTn). The nH for cardiac muscle contraction exceeds 1, both in humans[345,346] and in animals[315,347,348], indicative of a highly cooperative system (Figure 14).

5.2. Cooperativity of length-dependent activation

The thin-filament functional unit comprises seven actin monomers spanned by one tropomyosin dimer and one cTn complex (A_7TmTn).[136] Ca^{2+}-binding to cTnC promotes cTnI detachment from actin and potentiates tropomyosin movement

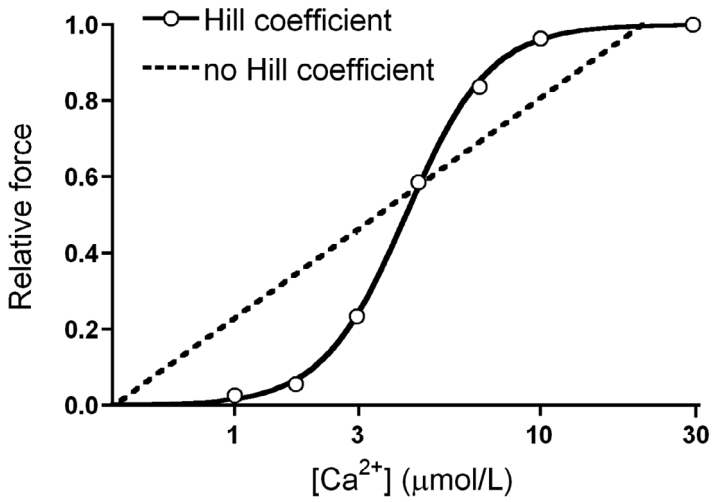

Figure 14. Cooperativity in cardiac muscle. In this plot of Ca^{2+} versus relative force the solid line depicts a unique cooperative relation between [Ca^{2+}] and force. The dashed line depicts a hypothetical system where cooperative activation is non-existent (x-axis here is non-logarithmic).

to expose myosin-binding sites on the surface of F-actin. This tropomyosin movement allows Ca^{2+}-cooperative activation of the thin-filament with additional recruitment of strong-binding myosin and actin monomers. Structural data suggest that individual strongly-bound cross-bridges bind to the regulatory unit (A_7TmTn spanning ~38.5 nm) and regulate tropomyosin movement up to ~3 units (covering ~115 nm) along the thin-filament, in the presence of Ca^{2+}.[139] This was also validated in biochemical studies.[241,349] Mounting evidence defines the role of tropomyosin in thin-filament Ca^{2+}-activation as three biochemical transitions. Tropomyosin increases "communication" between neighboring regulatory units, a property that is governed by the head-to-tail interaction (i.e., overlap region).[239-241,350,351] Removal of this overlap reduces cooperative-binding of myosin.[350-352]

Two recent studies support the idea that Ca^{2+}-cooperative effects are independent of myosin-binding and are strongly associated with the thin-filaments.[353,354] Sun et al.[353] reconstituted cardiac thin-filaments with fluorescent-labelled TnC and analyzed changes in the orientation of the structure of troponin. They observed that blebbistatin (which prevents strong-binding formation) had no effect on the Hill coefficient.[353] Farman et al.[354] reached similar conclusions, because in their experiments blebbistatin decreased Ca^{2+}-sensitivity and force, but did not affect the Hill coefficient. Importantly, the authors reconstituted rat cardiac thin-filaments with a cTnC mutant incapable of binding Ca^{2+} and observed that both Ca^{2+}-sensitivity and nH were decreased.[354] In addition, they observed that the effects of the cTnC mutant were greater at short (2.0 µm) than at longer (2.2 µm) sarcomere lengths. The authors attributed this result to

the ability of tropomyosin to recruit more regulatory units (A_7TmTn) upon stretching; tropomyosin stiffness increases at longer sarcomere length and affects up to three to four A_7TmTn units, whereas at shorter sarcomere length tropomyosin is less stiff and would only affect one or two A_7TmTn units.

Taken together, these data imply that cooperative activation via thin-filaments is partly responsible for the length-dependent behaviour. This is consistent with the observation of impaired Ca^{2+}-cooperative activation in muscle carrying cardiomyopathy-causing mutations in genes encoding cTnT.[259,355] Because tropomyosin overlap regions are required for proper formation of a ternary complex with the N-terminal tail of cTnT[356] (which in turn is essential to maintain the thin-filament in the B-state[357,358]), this suggests that troponin mutations disrupt the structure of the troponin-tropomyosin complex[359]. Also, impaired tropomyosin-tropomyosin interactions could decrease near-neighbour interactions and decrease length-dependent activation.

DISCUSSION

Over a century of research on the Frank-Starling Law has greatly advanced our knowledge of the fundamental basis of muscle. It mandates that, at the single cardiomyocyte level, there is a direct relation between sarcomere length and myofilament sensitivity to Ca^{2+} ions, such that more force is generated at a given concentration of Ca^{2+} as sarcomere length is increased. Although a unifying idea to explain how the myofilament "senses" length alterations remains in dispute, evidence supports the stretch-induced effects are central to length-dependent force changes. Also, changes in Ca^{2+}-activation upon muscle lengthening must be considered. This review has provided substantial evidence that myofilament length-dependent activation is a composite of several synergistically mechanokinetic processes. Length-dependent activation is the sum of increased Ca^{2+}-affinity of cTnC, alterations in interfilament lattice spacing, titin-induced stretch, and the formation of strong-binding cross-bridges, cTn complex changes, and Ca^{2+}-cooperative mechanisms.

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3

PERTURBED LENGTH-DEPENDENT ACTIVATION IN HUMAN HYPERTROPHIC CARDIOMYOPATHY WITH MISSENSE SARCOMERIC GENE MUTATIONS

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"Sometimes your language is too 'wooly' and it is at some points not entirely clear what you want to state."

Jolanda van der Velden

ABSTRACT

Rationale: High myofilament Ca^{2+}-sensitivity has been proposed as trigger of disease pathogenesis in familial hypertrophic cardiomyopathy (HCM) based on *in vitro* and transgenic mice studies. However, myofilament Ca^{2+}-sensitivity depends on protein phosphorylation and muscle length and at present data in human are scarce.

Objective: To investigate if high myofilament Ca^{2+}-sensitivity and perturbed length-dependent activation is characteristic for human HCM with mutations in thick and thin filament proteins.

Methods and results: Cardiac samples from HCM patients harboring mutations in genes encoding thick (*MYH7*, *MYBPC3*) and thin (*TNNT2*, *TNNI3*, *TPM1*) filament proteins were compared with sarcomere mutation-negative HCM (HCM_{smn}) and non-failing donors. Cardiomyocyte force measurements showed higher myofilament Ca^{2+}-sensitivity in all HCM samples and low phosphorylation of protein kinase A (PKA)-targets compared to donors. After exogenous PKA treatment, myofilament Ca^{2+}-sensitivity was either similar ($MYBPC3_{mut}$, $TPM1_{mut}$, HCM_{smn}), higher ($MYH7_{mut}$, $TNNT2_{mut}$) or even significantly lower ($TNNI3_{mut}$) compared to donors. Length-dependent activation was significantly smaller in all HCM than in donor samples. PKA treatment increased phosphorylation of PKA-targets in HCM myocardium and normalized length-dependent activation to donor values in HCM_{smn} and HCM with truncating *MYBPC3* mutations, but not in HCM with missense mutations. Replacement of mutant by wild-type troponin in $TNNT2_{mut}$ and $TNNI3_{mut}$ corrected length-dependent activation to donor values.

Conclusion: High myofilament Ca^{2+}-sensitivity is a common characteristic of human HCM and partly reflects hypophosphorylation of PKA-targets compared to donors. Length-dependent sarcomere activation is perturbed by missense mutations, possibly via post-translational modifications other than PKA-hypophosphorylation or altered protein-protein interactions, and represents a common pathomechanism in HCM.

NON-STANDARD ABBREVIATIONS AND ACRONYMS

HCM	Hypertrophic cardiomyopathy
MYH7 or β-MyHC	β-myosin heavy chain
MYBPC3 or cMyBP-C	Cardiac myosin-binding protein-C
TNNI3 or cTnI	Cardiac troponin I
TNNT2 or cTnT	Cardiac troponin T
TNNC1 or cTnC	Cardiac troponin C
TPM1 or α-Tm	α–tropomyosin
PKA	Protein kinase A
LV	Left ventricular
HCM_{mut}	Sarcomere mutation-positive HCM
HCM_{smn}	Sarcomere mutation-negative HCM
SEM	Standard error of the mean
F_{max}	Maximal developed force
ΔF_{max}	Difference in F_{max} between sarcomere lengths of 1.8 and 2.2 μm
EC_{50}	Ca^{2+}-sensitivity ($[Ca^{2+}]$ at which half of F_{max} is reached)
ΔEC_{50}	PKA-mediated or length-dependent change in myofilament Ca^{2+}-sensitivity
ssTnI	Slow skeletal troponin I
Ser	Serine
PTMs	Post-translational modifications
B-state	Blocked state
C-state	Ca^{2+}-induced state
M-state	Myosin-induced state

INTRODUCTION

Myofilament contraction is initiated by interaction between the thin actin and thick myosin filaments. This actin-myosin interaction (i.e. thin filament-myosin cross-bridge binding), and the magnitude of myofilament force generation is tightly regulated by muscle length, Ca^{2+}-binding and protein phosphorylation.[1] Defective proteins as a result of mutations in genes encoding sarcomeric proteins may directly impair regulation of muscle contraction and manifest themselves as phenotypic aberrations of the heart. Hypertrophic cardiomyopathy (HCM) reflects the pathological phenotype associated with sarcomeric gene mutations.[2, 3] Affecting 1:500 individuals worldwide, HCM is the most common cause of sudden death in young people.[2] Genotyping studies have identified a disease-causing mutation in ~70% of all HCM patients.[4] Mutations in thick filament-encoding genes, *MYH7* (β-myosin heavy chain, β-MyHC) and *MYBPC3* (cardiac myosin-binding protein-C, cMyBP-C) account for approximately 80% of all identified sarcomere mutations, while ~18% of the mutations are found in thin filament-encoding genes, *TNNI3* (cardiac troponin I, cTnI),*TNNT2* (cardiac troponin T, cTnT), *TNNC1* (cardiac troponin C, cTnC), *TPM1* (α–tropomyosin, α-Tm) and *ACTC1* (α-cardiac actin).[5] The remaining 2% is attributed to incidental mutations in the thick filament genes, *MYL3* and *MYL2,* encoding the regulatory and essential myosin light chain, and the sarcomere-associated gene *TTN*, which encodes titin.[5] Based on the numerous sarcomeric gene mutations HCM is referred to as "disease of the sarcomere".

Both animal[6, 7] and clinical[8-10] studies have shown that carriers of HCM-causing mutations demonstrate early signs of cardiac dysfunction, even before a hypertrophic phenotype is observed. Additionally, sudden cardiac death occurs in young individuals in the absence of clinically detectable cardiac hypertrophy.[11] This suggests that the initial defects in cardiac performance are triggered by mutant sarcomeric proteins rather than remodeling of the heart. Sarcomere mutations may directly impair myofilament function and contractile performance of the heart,[3] or indirectly via changes in intracellular Ca^{2+}-handling.[12] As the myofilaments represent a major intracellular buffer of Ca^{2+}, any perturbation in myofilament Ca^{2+}-sensitivity may provide a substrate for cardiac arrhythmias. Only recently, the group of Knollmann[13] showed that *TNNT2* mutations alter intracellular Ca^{2+}-handling via myofilament Ca^{2+}-sensitization in transgenic mice models, which was associated with altered action potential regulation and occurrence of arrhythmias. This implies that myofilament Ca^{2+}-sensitivity is central in HCM pathology.

Whether or not myofilament sensitization to Ca^{2+} is of relevance in human HCM is a matter of ongoing research.[14-16] Our recent studies in manifest HCM with *MYBPC3* mutations showed higher myofilament Ca^{2+}-sensitivity compared to non-failing donor myocardium.[15, 16] However, high Ca^{2+}-sensitivity coincided with low phosphorylation of target proteins of the β-adrenergic signaling pathway, cTnI and cMyBP-C, and was normalized to donor values by exogenous protein kinase A (PKA) treatment.[15] This suggests that the high myofilament Ca^{2+}-sensitivity observed in human HCM

with *MYBPC3* mutations is due to secondary disease-related post-translational modifications rather than the mutation itself. In addition to post-translational protein modifications, muscle length represents an important determinant of myofilament Ca^{2+}-sensitivity. Recently, we observed impairment of length-dependent sarcomere activation evident from a blunted length-dependent increase in myofilament Ca^{2+}-sensitivity in HCM with *MYBPC3* mutations.[15] Under normal conditions intracellular Ca^{2+} buffering will increase with increased myofilament Ca^{2+}-sensitivity upon myofilament lengthening.[17, 18] Perturbations in length-dependent myofilament activation will alter Ca^{2+} buffering by the sarcomeres and may provide a basis for altered Ca^{2+}-handling in HCM.

In the present study, we investigated if perturbed length-dependent activation rather than high myofilament Ca^{2+}-sensitivity is a common pathomechanism in human HCM with mutations in thick and thin filament genes. Measurements in sarcomere mutation-positive HCM (HCM_{mut}) were compared with sarcomere mutation-negative HCM (HCM_{smn}) and non-failing donors. Our study shows that mutation-related changes in myofilament Ca^{2+}-sensitivity are diverse and depend on the affected gene. We observed impaired length-dependent sarcomere activation in all HCM samples. The blunted length-dependence of sarcomeres was due to low phosphorylation of PKA targets compared to donor myocardium in patients with a *MYBPC3* truncating mutations and HCM_{smn}, but a direct consequence of HCM missense mutations. Using troponin exchange in HCM cardiomyocytes with a homozygous *TNNT2* and a heterozygous *TNNI3* mutation, we provide evidence that less than 50% of poison peptide is sufficient to impair length-dependent activation of the sarcomeres. Since most patients carry a heterozygous sarcomere mutation that may result in relatively low levels of mutant (poison) peptide, impaired length-dependent sarcomere activation represents a pathomechanism in HCM.

METHODS

An expanded version of the Methods section can be found in the online-only Data Supplement.

Myocardial samples

Left ventricular (LV) septum tissue was obtained from HCM patients harboring thick and thin filament gene mutations during myectomy surgery to relieve LV outflow obstruction. Hypertrophic obstructive cardiomyopathy was evident from the high septal thickness (normal value <13 mm) and high LV outflow tract pressure gradient (normal value <30 mm Hg). Clinical characteristics and mutation information of HCM patients are listed in Table 1. Our study included patients carrying heterozygous mutations in *MYBPC3* (n=21; $MYBPC3_{mut}$), *MYH7* (n=6; $MYH7_{mut}$), *TNNI3* (n=2; $TNNI3_{mut}$) and *TPM1* (n=1; $TPM1_{mut}$). The $MYBPC3_{mut}$ group consisted of patients with truncating (n=17) and missense (n=4) mutations. Data for these two $MYBPC3_{mut}$

groups are presented separately. Septum tissue was also obtained from 1 end-stage failing HCM patient carrying a homozygous *TNNT2* mutation (*TNNT2*$_{mut}$). Septum myectomy tissue from 7 HCM patients in whom no mutation was found after screening of 8 genes (sarcomere mutation-negative HCM; HCM$_{smn}$) and cardiac tissue from 12 non-failing donors served as controls. Donors (age range from 14 to 65 years; mean 39±5 years; 9/3 male/female, respectively) had no history of cardiac abnormalities, normal ECG and normal ventricular function on echocardiography within 24 hours of heart transplantation. Samples were obtained after written informed consent and the study protocol was approved by the local ethical committees.

Isometric force measurements

Small cardiac tissue samples were thawed in relaxing solution (5.95 mM Na_2ATP, 6.04 mM $MgCl_2$, 2 mM EGTA, 139.6 mM KCl, 10 mM Imidazole, pH 7.0) and cardiomyocytes were mechanically isolated by tissue disruption. Cardiomyocytes were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a piezoelectric motor.[15] Isometric force measurements were performed at maximal and submaximal [Ca^{2+}] (ranging from 1 to 30 µmol/L) and sarcomere lengths of 1.8 and 2.2 µm (Online Figures IA and IB). Average sarcomere lengths were determined by means of a spatial Fourier transformation as described previously.[19] Passive force (F_{pas}) was determined by shortening the myocyte in a relaxing solution (10^{-9} µmol/L) by 30% of its length. Maximal developed force (F_{max}) was determined by activating the cardiomyocyte at saturating [Ca^{2+}] (30 µmol/L), generating a total force value (F_{total}). F_{max} was obtained by subtracting F_{pas} from F_{total} (i.e. $F_{max}=F_{total}-F_{pas}$). Maximal tension (in kN/m^2) was calculated as F_{max} normalized to cross-sectional area of the cardiomyocytes. Force-Ca^{2+} relations were fit to a modified Hill equation and myofilament Ca^{2+}-sensitivity was denoted as EC_{50} ([Ca^{2+}] at which half of F_{max} was reached). The length-dependent increase in myofilament Ca^{2+}-sensitivity upon an increase in sarcomere length is based on the difference in EC_{50} at sarcomere lengths of 1.8 and 2.2 µm (ΔEC_{50}). Additional force measurements were performed following exogenous PKA treatment of cells for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U/incubation, Sigma).

Exchange of recombinant human wild-type troponin complex in single cardiac cells

Preparation of recombinant human wild-type troponin complex

Expression of cDNA encoding human wild-type cardiac troponin subunits (cTnC, myc-tag labeled cTnT (cTnT-myc), cTnI), purification and reconstitution were performed as described previously.[20]

Troponin exchange protocol

Single cardiomyocytes from the *TNNT2*$_{mut}$ heart and one of the *TNNI3*$_{mut}$ hearts were mechanically isolated by tissue disruption in ice-cold rigor solution (132 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris, 5 mM EGTA, 1 mM NaAzide, pH 7.1). Cardiomyocytes were chemically permeabilized by incubation for 5 minutes in rigor solution containing 0.5% (v/v) Triton-X100. After permeabilization, cells were washed twice with rigor solution followed by washing in exchange solution (10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT) (pH 6.9). Subsequently, single cardiomyocytes were incubated overnight at 4°C in exchange solution containing the appropriate concentration of recombinant human troponin complex (0.25, 0.5 and 1.0 mg/mL) with the addition of 4 mM $CaCl_2$, 4 mM DTT, 5 µl/mL protease inhibitor cocktail (PIC, Sigma, P8340) and 10 µl/mL phosphatase inhibitor cocktail 2 and 3 (PhIC, Sigma, P2850, P5726) (pH 6.9). The next day, cells were washed twice in rigor solution followed by washing in relaxing solution. Our previous study showed a homogenous distribution of recombinant troponin complex in cardiomyocytes using this exchange protocol.[21]

Determination of troponin exchange percentage

Half of the cardiomyocyte suspension was used for isometric force measurements, whereas the other half was used to analyze troponin exchange percentage. This half was treated with 2D-clean-up kit (GE Healthcare), homogenized in sample buffer (15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT) and protein concentration was measured with *RCDC* Protein Assay kit II (Biorad) as described previously.[20]

To determine the degree of exchange of endogenous mutant troponin by recombinant wild-type cardiac troponin Western blotting was performed. Recombinant wild-type cTnT was labeled with a myc-tag, which allowed differentiation between endogenous and recombinant cardiac troponin complex. Proteins were separated on a 1D SDS-PAGE and blotted onto a nitrocellulose membrane. A specific monoclonal antibody was used against cTnT (Clone JLT-12, Sigma) to detect endogenous and recombinant cTnT by chemiluminescence (ECL, Amersham Biosciences) as described previously.[20]

Myofilament protein phosphorylation

SYPRO Ruby and ProQ-Diamond staining of gradient gels

Myofilament protein phosphorylation levels in HCM and donor myocardium and in PKA-treated samples (100 U/mL relaxing solution) were analyzed on 4-15% pre-cast Tris-HCl gels (BioRad) and stained with SYPRO Ruby and ProQ-Diamond phosphostain as described previously.[22] Phosphorylation of cMyBP-C and cTnI was normalized to SYPRO-stained cMyBP-C and cTnI, respectively. Protein phosphorylation values were normalized to the values found in untreated donors, which were set to 1.

Table 1. Mutations and clinical characteristics of patients.

	Mutation	Type	Age	Sex	LVOT	ST
***MYBPC3*$_{mut}$**		Truncating mutations				
1	c.927-2A>G	splice site	37	M	61	19
2	c.927-2A>G	splice site	48	M	82	18
3	c.927-2A>G	splice site	22	M	71	30
4	c.1458-1G>C	splice site	41	F	92	22
5	c.2373duplicationG	insertion	32	F	100	30
6	c.2373duplicationG	insertion	39	F	60	20
7	c.2373duplicationG	insertion	45	F	94	20
8	c.2373duplicationG	insertion	62	M	64	23
9	c.2373duplicationG	insertion	44	F	100	17
10	c.2373duplicationG	insertion	69	M	74	19
11	c.2373duplicationG	insertion	57	F	74	24
12	c.2373duplicationG	insertion	32	M	88	23
13	c.2373duplicationG	insertion	60	M	77	23
14	c.2864.2865delCT	deletion	42	M	116	23
15	c.2864.2865delCT	deletion	45	M	100	20
16	c.2864.2865delCT	deletion	62	F	67	15
17	c.3407.3409del	deletion	55	M	UK	UK
***MYBPC3*$_{mut}$**		Missense mutations				
18*	p.E258K	missense	35	M	4	27
19	p.E258K	missense	45	M	72	24
20	p.G531R	missense	51	M	110	22
21	p.R597Q	missense	47	F	85	20
***MYH7*$_{mut}$**						
1	p.R403Q	missense	25	M	85	34
2	p.V606M	missense	46	F	79	17
3	p.S782R	missense	30	F	128	29
4	p.R787H	missense	61	M	UK	UK
5	p.T1377M	missense	58	F	100	20
6	p.T1377M	missense	43	M	UK	UK
***TNNT2*$_{mut}$**						
1	p.K280N	missense	26	M	UK	UK
***TNNI3*$_{mut}$**						
1	p.R145W	missense	46	M	100	23
2	p.R145W	missense	66	M	100	16
***TPM1*$_{mut}$**						
1	p.I284V	missense	65	M	100	16

Table 1. (Continued)

	Mutation	Type	Age	Sex	LVOT	ST
HCM_{smn}	Sarcomere mutation negative					
1			46	F	105	24
2			57	M	117	25
3			75	F	137	20
4			72	F	88	24
5			65	F	85	19
6			49	M	61	20
7			46	M	81	19

LVOT, left ventricular outflow tract pressure gradient in mmHg; ST, septal thickness in mm; p, protein amino residue; c, codon region; del, deletion; UK, unknown. *Patient was operated due to extreme hypertrophy and small LV cavity.

Western blot analysis of cMyBP-C phosphorylation at PKA sites

Phosphorylation of the cMyBP-C PKA sites Ser275 and Ser284 was assessed using phosphospecific antibodies in Western blots.[15]

Phos-Tag acrylamide gels

Phos-Tag™ acrylamide gels were performed to visualize phosphorylated cTnI species using alkoxide-bridged dication manganese (Mn^{2+}) complex as phosphate-binding tag (Phos-tag) molecule. Mn^{2+}-Phos-Tag molecules specifically bind phosphorylated proteins and as a result, their migration speed is highly reduced. Non-phosphorylated and phosphorylated cTnI species were separated in 1D PAGE with polyacrylamide-bound Mn^{2+}-Phos-Tag, transferred to Western blots and probed with anti-cTnI monoclonal antibody (8I-7 Spectral Diagnostics).[23]

Data analysis

Data analysis and statistics were performed using Prism version 4.0 (Graphpad Software, Inc., La Jolla, CA) and SPSS version 15.0 (IBM, Armonk, NY). Data are presented as mean±SEM of all single cardiomyocytes per patient group (8 groups, i.e. the 6 HCM sarcomere mutation positive groups (HCM_{mut}), HCM_{smn} and non-failing donor). To take into account the repeated sample assessments within patient/donor groups multilevel analysis was performed. Comparison between all groups was performed for Ca^{2+}-sensitivity at 2.2 μm sarcomere length and length-dependent activation of cardiomyocytes before and after PKA. Paired-group comparisons were performed for F_{max} at 1.8 and 2.2 μm sarcomere length before and after PKA.

All data was tested for normality using the Shapiro-Wilk Test. Normality was assumed when $p>0.05$ and the variances were equal. When assumption of normality was violated the data set was logarithmic-transformed and normality re-tested.[24] Detailed information on statistical analyses of the data presented in Figures 1-2 and

5 and Table 2 of the manuscript are presented in the Online Tables III to X. To take differences in group size into account multilevel analysis was performed (Online Tables III to VII and X to XI). Two data sets were expressed as logarithmic-transformed groups (Online Tables III and V). To check for paired-group differences on F_{max} between sarcomere lengths of non-treated (Online Table VIII) or treated cardiomyocytes with PKA (Online Table IX), paired-samples t-Test was conducted. $p<0.05$ was considered significant for both tests and 95% confidence intervals (CI) were calculated to gain insight into the range of the mean differences between and among groups. In case of logarithmic transformed data, a set of 95% CIs was calculated by taking the exponential of the natural logarithmic values (Online Tables III and V). The CIs reflect ratios stating that the mean difference between two groups can be [x to y] time higher or lower than the group of comparison.

RESULTS

Myofilament Ca^{2+}-sensitivity in HCM compared to non-failing donors

To assess myofilament Ca^{2+}-sensitivity, force-Ca^{2+} relations were constructed for all cardiac samples at a sarcomere length of 2.2 μm. Ca^{2+}-sensitivity for all HCM samples was significantly higher compared to donors, based on their lower EC_{50} values (Figure 1A). As the high Ca^{2+}-sensitivity may be due to low phosphorylation levels of PKA-target proteins, measurements were repeated after PKA treatment. The PKA-induced reduction in Ca^{2+}-sensitivity (ΔEC_{50}) was larger in all HCM groups compared to donors (with significant changes in *MYBPC3*$_{mut}$, *MYH7*$_{mut}$ and *TNNI3*$_{mut}$), except for the *TNNT2*$_{mut}$ which only showed a minor non-significant change (Figure 1B). After PKA treatment myofilament Ca^{2+}-sensitivity was close to donor values in *MYBPC3*$_{mut}$, *TPM1*$_{mut}$ and HCM$_{smn}$, while Ca^{2+}-sensitivity remained significantly higher compared to donor in *MYH7*$_{mut}$ and *TNNT2*$_{mut}$ (Figure 1C). Interestingly, after PKA Ca^{2+}-sensitivity was significantly lower in *TNNI3*$_{mut}$ compared to HCM$_{smn}$ and donor.

F_{max} at a sarcomere length of 2.2 μm was significantly lower in all HCM groups compared to donor and was not corrected by PKA treatment (Online Table I). In addition, the steepness of the force-Ca^{2+} relation was significantly lower and not corrected by PKA in all HCM groups compared to donors (data not shown).

Length-dependent myofilament force characteristics

Force development was measured at various [Ca^{2+}] and two sarcomere lengths to determine length-dependent activation of myofilaments (Figure 2A). Following a sarcomere length increase from 1.8 to 2.2 μm at maximal Ca^{2+}-activation, donor samples showed a significant increase in F_{max} (Table 2), which is in accordance with the well-known effects of length on force development.[25] A significant length-dependent increase in F_{max} was observed in *MYBPC3*$_{mut}$, *MYH7*$_{mut}$, *TPM1*$_{mut}$ and HCM$_{smn}$ heart samples, while no significant increase in F_{max} was found for both *TNNT2*$_{mut}$ and *TNNI3*$_{mut}$. As illustrated in Table 2 the ΔF_{max} (difference in F_{max} between sarcomere

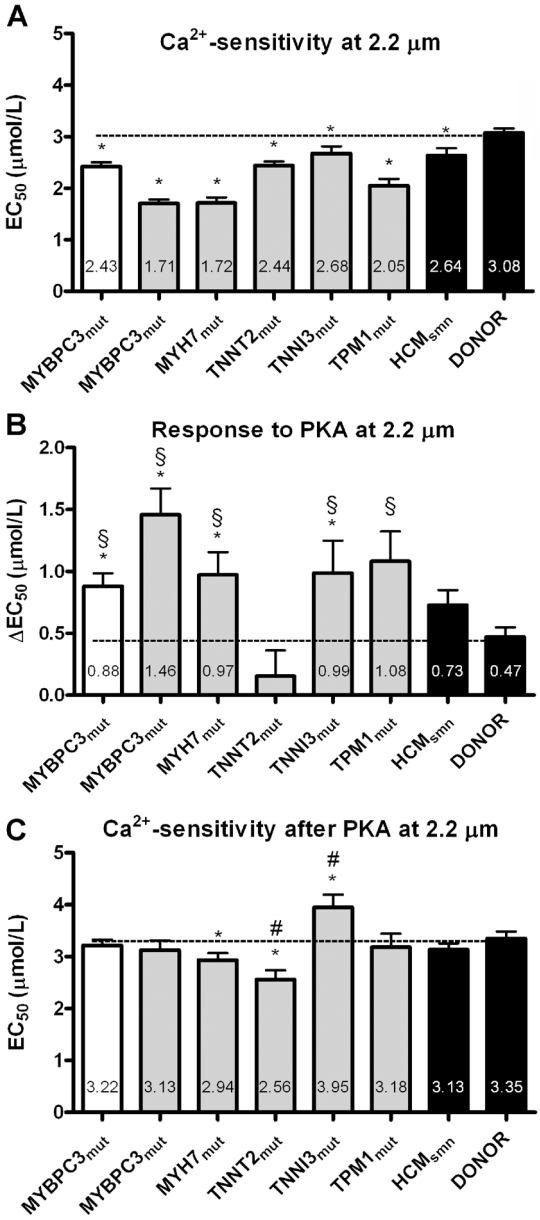

Figure 1. Myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm. A. Myofilament Ca^{2+}-sensitivity (EC_{50}) was significantly higher in all HCM groups compared to donors. **B.** The PKA-induced reduction (ΔEC_{50}) in myofilament Ca^{2+}-sensitivity was larger in HCM groups compared to donors, except in the *TNNT2*$_{mut}$ sample, in which PKA had no significant effect. **C.** Myofilament Ca^{2+}-sensitivity was similar in *MYBPC3*$_{mut}$, *TPM1*$_{mut}$, HCM_{smn} and donor after treatment with PKA, while it was higher than donor in *MYH7*$_{mut}$ and *TNNT2*$_{mut}$. PKA-treated *TNNI3*$_{mut}$ cells showed a lower myofilament Ca^{2+}-sensitivity compared to HCM_{smn} and donor. Open bar graph represents *MYBPC3* truncating mutation; Closed gray bar graphs represent missense mutations. $^{*}p<0.05$ versus donor; $^{§}p<0.05$ versus *TNNT2*$_{mut}$; $^{\#}p<0.05$ versus HCM_{smn}.

lengths of 1.8 and 2.2 μm) was much lower in HCM with missense mutations compared to the other groups.

To investigate the length-dependent increase in myofilament Ca^{2+}-sensitivity, force at submaximal [Ca^{2+}] was normalized to the maximal developed force to obtain normalized force-Ca^{2+} relations at both sarcomere lengths (Figure 2B). For all HCM and donor groups, the normalized force-Ca^{2+} relation shifted to the left as sarcomere length increased, indicative for increased Ca^{2+}-sensitivity. However, the increase in myofilament Ca^{2+}-sensitivity upon an increase in sarcomere length (ΔEC_{50}) was significantly lower in all HCM_{mut} and HCM_{smn} samples compared to donors (Figure 2C).

Stimulation of the β-adrenergic receptor pathway has been shown to enhance the length-dependent shift in the force-Ca^{2+} relation, suggesting a modulating role for PKA-mediated protein phosphorylation in length-dependent sarcomere activation.[26, 27] As previous studies showed lower phosphorylation levels of myofilament PKA-target proteins in HCM compared to donors,[15, 16] the blunted length-dependent activation in HCM compared to donors may be explained by a difference in protein phosphorylation level. Indeed, analysis of protein phosphorylation showed lower phosphorylation levels of both cTnI and cMyBP-C in all HCM groups compared to non-failing donors, except in $TNNT2_{mut}$ in which phosphorylation of the PKA-target proteins did not differ from donor (Figure 3A).

To test if the blunted length-dependent change in F_{max} and myofilament Ca^{2+}-sensitivity was corrected by PKA-mediated phosphorylation of cMyBP-C and cTnI, force measurements were performed after pre-treatment with exogenous PKA in a subset of HCM and donor samples. PKA pre-treated $MYBPC3_{mut}$, $MYH7_{mut}$, HCM_{smn} and donor cells showed a significant increase in maximal force following a sarcomere length increase, similar as observed in non-treated cardiomyocytes (Table 2). PKA did not restore the blunted length-dependent increase in F_{max} evident from the significantly lower ΔF_{max} in HCM with missense mutations compared to donor (Table 2). Pre-treatment with exogenous PKA significantly enhanced the length-dependent shift in myofilament Ca^{2+}-sensitivity in HCM with truncating $MYBPC3_{mut}$ and HCM_{smn} samples to values observed in donors, but did not correct the blunted length-dependent change in EC_{50} in all other HCM mutant groups harboring missense mutations (Figure 2D).

As the $MYBPC3_{mut}$ and $MYH7_{mut}$ groups consisted of different mutations, we averaged functional data for each HCM mutation separately. Data are shown in the supplemental material (Online Table II).

PKA-mediated protein phosphorylation

To determine if absence of an effect of PKA on sarcomere functional properties is due to a defect in PKA-mediated protein phosphorylation in HCM myocardium, phosphorylation status of PKA-target proteins was analyzed in HCM and donor samples incubated with exogenous PKA. Figure 3B shows HCM samples incubated without and with PKA separated by 1D-gel electrophoresis and stained with ProQ-

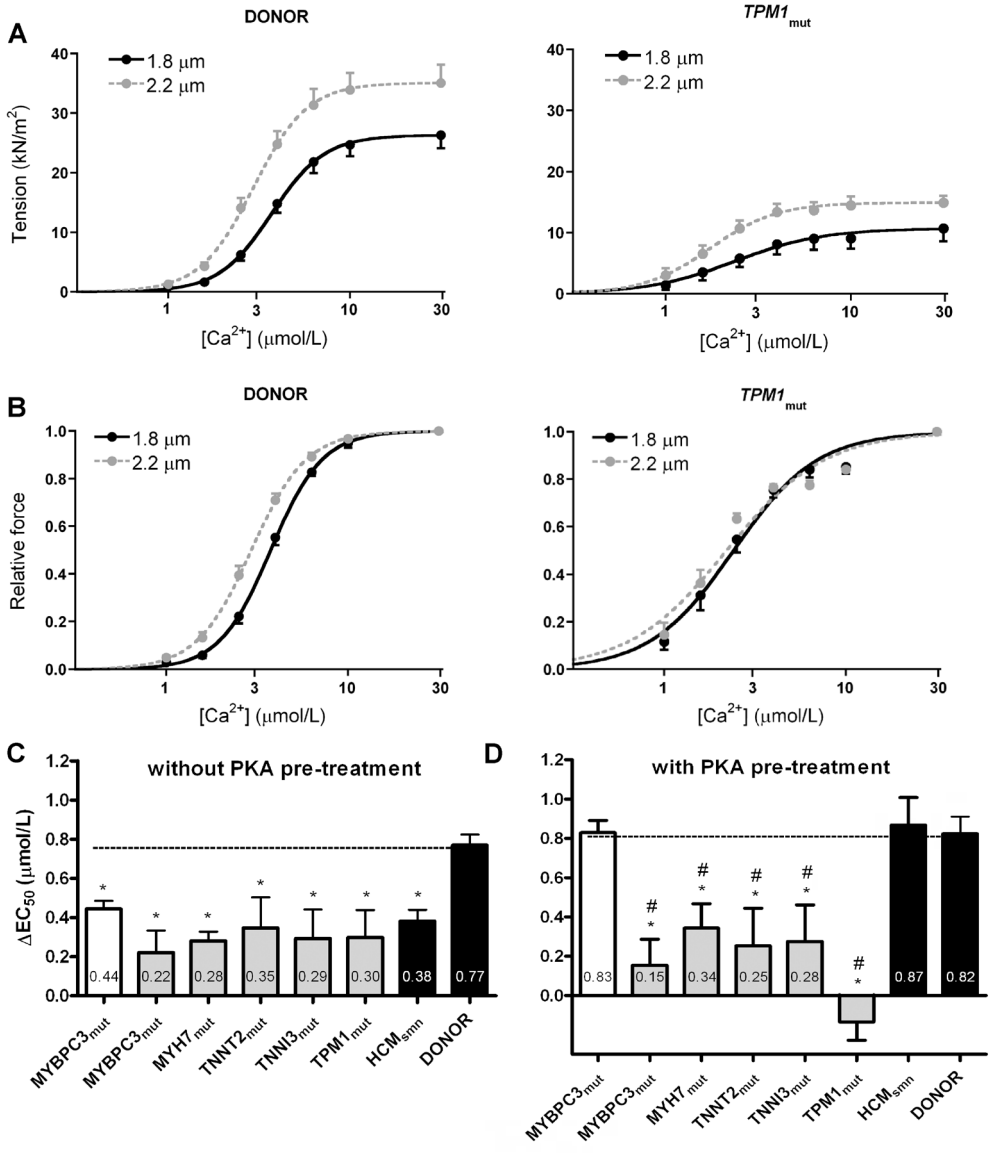

Figure 2. Myofilament length-dependent activation. A. Tension development as a function of [Ca^{2+}] at short (1.8 μm) and long (2.2 μm) sarcomere length for donor (left) and *TPM1*$_{mut}$ (right) heart samples. **B.** Normalized force-Ca^{2+} relationships for donor (left) and *TPM1*$_{mut}$ (right) heart samples. **C.** The length-dependent increase in myofilament Ca^{2+}-sensitivity was lower in all HCM$_{mut}$ compared to donors before PKA treatment. **D.** PKA pre-treatment restored length-dependent activation to donor in HCM with truncating *MYBPC3*$_{mut}$ and HCM$_{smn}$, but not in HCM$_{mut}$ with missense mutations. Open bar graph represents *MYBPC3* truncating mutations; Closed gray bar graphs represent missense mutations.*p<0.05 versus donor; $^{\#}$p<0.05 versus HCM$_{smn}$.

Table 2. Effects of sarcomere length increase on F_{max} before and after PKA.

	no **PKA pre-treatment**				**PKA pre-treatment**			
	F_{max} (kN/m^2)				**F_{max}** (kN/m^2)			
Sample	1.8 µm	2.2 µm	ΔF_{max}	N/n	1.8 µm	2.2 µm	ΔF_{max}	N/n
Truncating								
MYBPC3$_{mut}$	19.6±1.5	29.8±2.4**	10.2±1.2	N=14 n=47	27.6±1.5	37.0±2.0**	9.4±1.3	N=4 n=19
Missense								
MYBPC3$_{mut}$	13.0±1.3	15.5±1.7**	2.5±0.8*	N=4 n=12	11.4±1.0	13.9±1.5**	2.5±1.0*	N=4 n=12
MYH7$_{mut}$	15.1±1.6	19.1±1.8**	3.9±1.0*	N=6 n=32	18.8±2.6	22.6±3.1**	3.8±1.1*	N=4 n=15
TNNT2$_{mut}$	21.5±3.2	24.4±6.0	3.0±4.4	N=1 n=6	13.7±2.1	15.2±2.4	1.5±1.3*	N=1 n=4
TNNI3$_{mut}$	9.8±2.5	10.1±2.8	0.3±1.1*	N=2 n=8	8.4±1.4	9.5±1.9	0.83±0.8*	N=2 n=10
TPM1$_{mut}$	10.7±2.1	17.0±3.0**	6.3±1.5	N=1 n=6	9.1±2.3	9.6±1.7	0.58±0.7*#	N=1 n=4
HCM$_{smn}$	18.2±1.4	28.3±2.6**	10.1±1.7	N=7 n=31	26.8±2.3	38.7±2.7**	12.0±1.4	N=3 n=12
DONOR	26.3±2.2	35.1±3.1**	8.8±2.1	N=9 n=32	26.2±1.4	36.2±1.9**	10.0±1.6	N=3 n=12

$p<0.05$ was considered significant; *versus donor; N= number of samples; n= number of cardiomyocytes; **1.8 µm versus 2.2 µm; #ΔF_{max} (no PKA pre-treatment) versus ΔF_{max} (PKA pre-treatment).

Diamond and SYPRO Ruby. PKA increased phosphorylation of both PKA-targets, cMyBP-C and cTnI, in HCM samples. Phosphorylation levels (normalized to untreated donor samples, which were included on the gel and set to 1; dotted line) are depicted in Figure 3C and show that phosphorylation of cMyBP-C after PKA was close to values observed in donor samples in all HCM samples.

Analysis of phosphorylation at specific PKA sites (Ser275 and Ser284) on cMyBP-C confirmed increased PKA-mediated phosphorylation in HCM samples (Figure 4). In addition to cMyBP-C, phosphorylation of cTnI increased by PKA in all HCM samples (Figure 3B), although values did not reach the level found in donor myocardium (Figure 3C). PKA treatment of donor samples increased cTnI phosphorylation by ~25%. Similar data were obtained when cTnI phosphorylation was analyzed by Phos-Tag gel electrophoresis creating a pattern of un-, mono- and bisphosphorylated cTnI (Online Figure II). PKA-treated HCM samples showed increased bisphosphorylated cTnI levels, but some monophosphorylated cTnI remained. Previous Phos-Tag analysis of the donor samples (n=12) revealed a distribution pattern of 7% unphosphorylated,

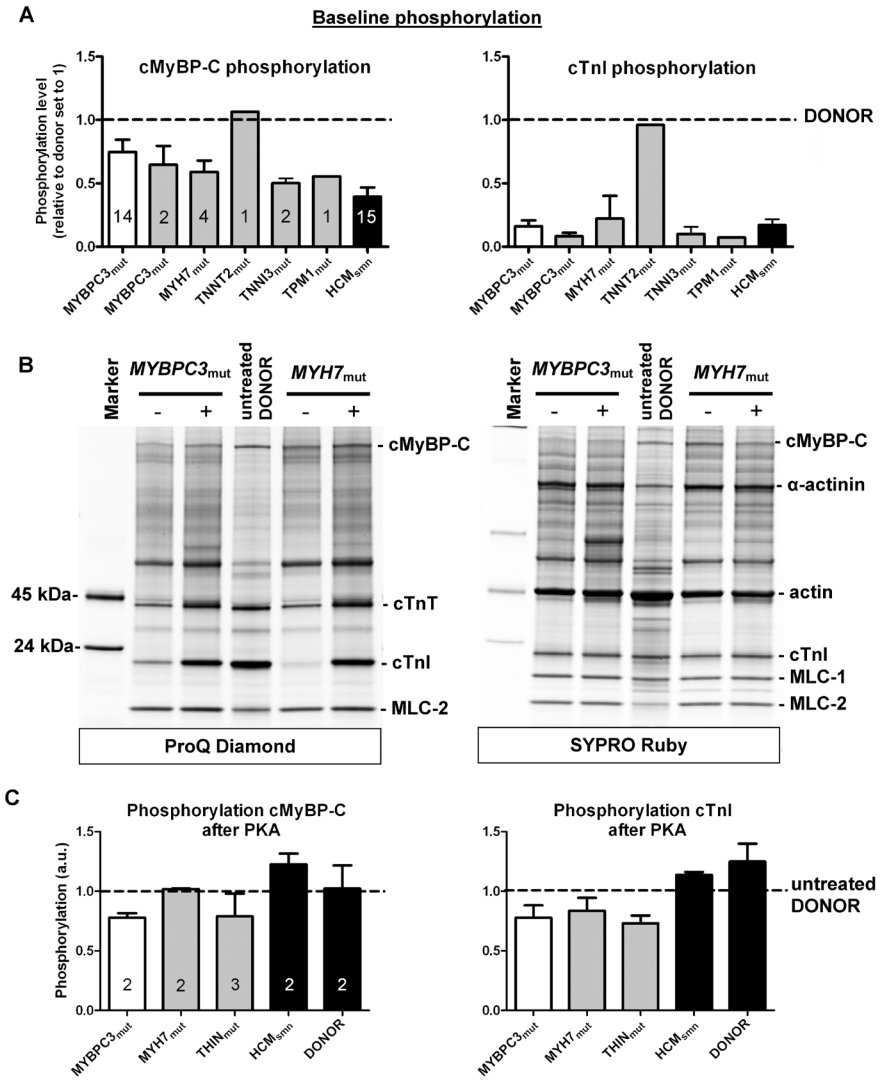

Figure 3. Phosphorylation of PKA-target proteins before and after PKA treatment. A. Protein phosphorylation values were corrected by the corresponding SYPRO-stained protein bands and normalized to the values found in donors, which were set to 1 (dotted line). Phosphorylation of cMyBP-C (left) and cTnI (right) was lower in all HCM samples compared to donors, except for the $TNNT2_{mut}$ sample, which showed relatively high phosphorylation. Open bar graph represents truncating MYBPC3 mutations; Closed gray bar graphs represent missense mutations. Phosphorylation of PKA-target proteins before and after PKA treatment. B. Cardiac samples before (-) and after (+) PKA treatment separated by 1D-gel electrophoresis and stained with ProQ-Diamond (phosphorylation) and SYPRO Ruby (total protein stain). **C.** Thin filament mutations were clustered in a single group ($THIN_{mut}$). ProQ-stained protein phosphorylation values of PKA-treated samples were corrected by the corresponding SYPRO-stained protein bands and normalized to values in non-treated donor samples, which were included on the gel and set to 1 (dotted line). PKA increased phosphorylation of both target proteins in all HCM groups. Numbers of samples included in the analyses are indicated in the bar graphs.

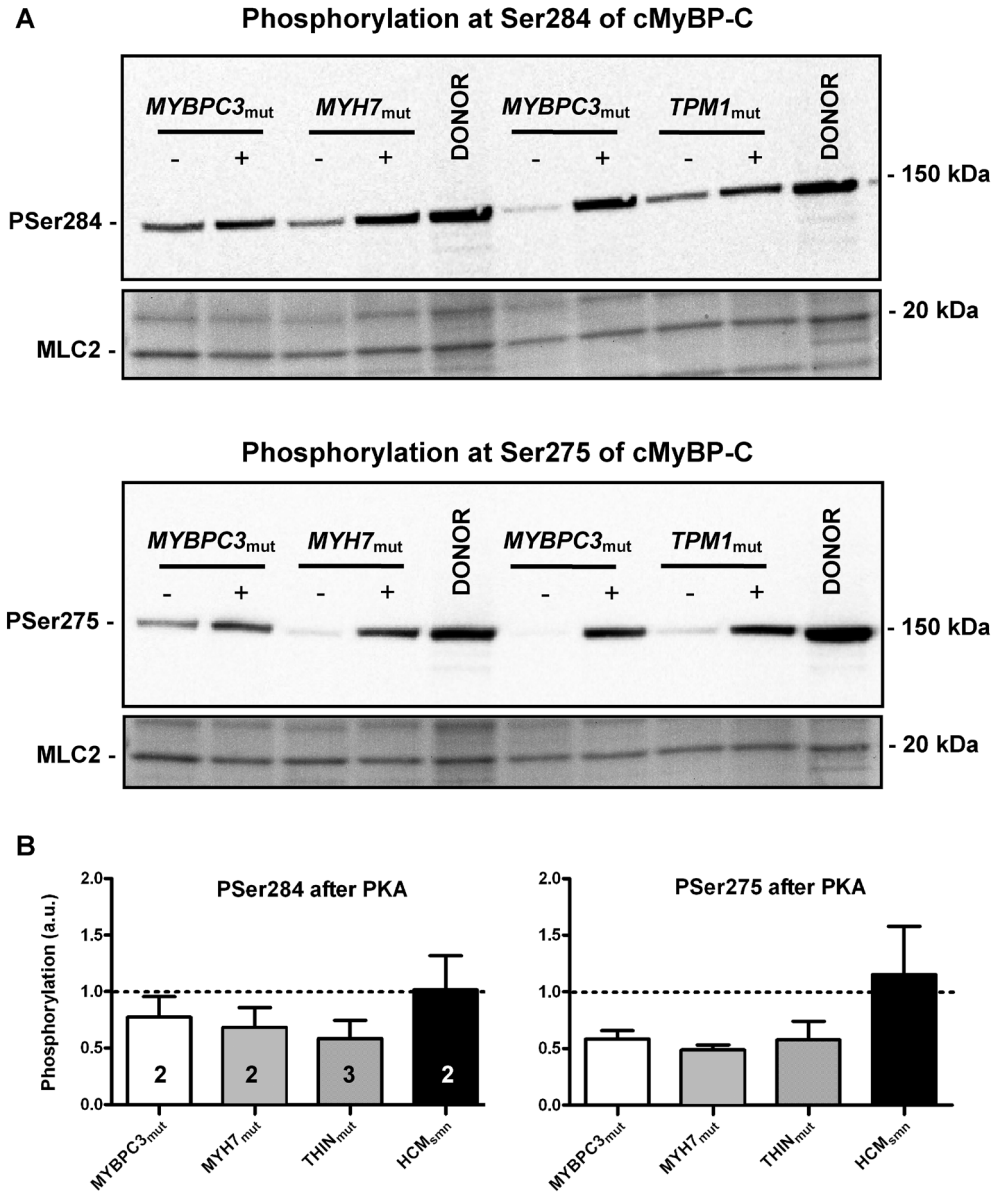

Figure 4. Site-specific phosphorylation of PKA-target sites of cMyBP-C. A. Western blot analysis of cMyBP-C phosphorylation with specific antibodies for PKA sites Ser284 (upper panel) and Ser275 (lower panel) before (-) and after (+) PKA treatment (phosphorylation values were corrected for minor differences in protein loading by Ponceau-stained MLC2, myosin light chain 2). **B.** Protein phosphorylation values were normalized to the values found in untreated donor samples, which were included on the blot and set to 1 (dotted line). Thin filament mutations were clustered in a single group ($THIN_{mut}$). Numbers of samples included in the analyses are indicated in the bar graphs. All samples showed an increased phosphorylation at both sites upon PKA treatment.

27% monophosphorylated and 66% bisphosphorylated cTnI.[15] Upon treatment of donor samples (n=2) with PKA cTnI was mostly bisphosphorylated (Online Figure II).

Correction of length-dependent activation in HCM with mutant cTnT and mutant cTnI by human recombinant wild-type troponin

To determine if mutant sarcomeric protein is the direct cause of the blunted length-dependent increase in myofilament Ca^{2+}-sensitivity, we performed troponin exchange experiments in cardiomyocytes from the *TNNT2*$_{mut}$ sample. The homozygous *TNNT2*$_{mut}$ necessarily results in 100% mutant cTnT and as such represents a unique tool to assess the level at which mutant protein perturbs sarcomere function. Exchange with increasing concentrations of wild-type human troponin complex (0.25, 0.5 and 1 mg/mL in the exchange solution) resulted in 62±2%, 78±1% and 86±1% troponin exchange based on Western blot analyses of endogenous and myc-tag labeled wild-type cTnT (Figure 5A; left blot). In exchanged cells without PKA pre-treatment, replacement of mutant troponin with unphosphorylated recombinant wild-type troponin did not restore the reduced length-dependent activation to donor values (Figure 5B). However, replacement of endogenous mutant troponin by unphosphorylated recombinant troponin reduces cTnI phosphorylation in the *TNNT2*$_{mut}$ cells. Therefore, measurements were also performed in the troponin exchanged cells after PKA treatment to increase cTnI phosphorylation to donor levels.

In cells in which 62% of mutant troponin was replaced by wild-type troponin the length-dependent increase in myofilament Ca^{2+}-sensitivity was still significantly lower compared to donor values, indicating that 38% of mutant protein is sufficient to impair length-dependent activation. However, the blunted length-dependent activation was restored to donor values in *TNNT2*$_{mut}$ exchanged cells harboring 22% and 14% endogenous mutant cTnT (Figure 5C). Similarly, exchange of mutant cTnI in one of the HCM samples harboring the R145W missense mutation in *TNNI3* by ~90% unphosphorylated wild-type troponin complex (Figure 5A; right blot) corrected length-dependent activation only after PKA treatment (Figures 5D, E). Hence, normalization of length-dependent activation to donor values upon exchange with wild-type cTn complex is only evident after PKA treatment of troponin-exchanged cells.

DISCUSSION

Our study shows that high myofilament Ca^{2+}-sensitivity in human HCM myocardium is independent of the presence of a sarcomere mutation and at least partly explained by protein hypophosphorylation. Sarcomere mutations may modify Ca^{2+}-sensitivity, but the direction and magnitude of the change depends on the affected gene. Impaired length-dependent activation of sarcomeres represents a common pathomechanism underlying HCM, and could not be corrected by PKA treatment in HCM with missense mutations in genes encoding thick and thin filament proteins. Moreover, our troponin exchange experiments provide direct proof that mutant troponin impairs length-

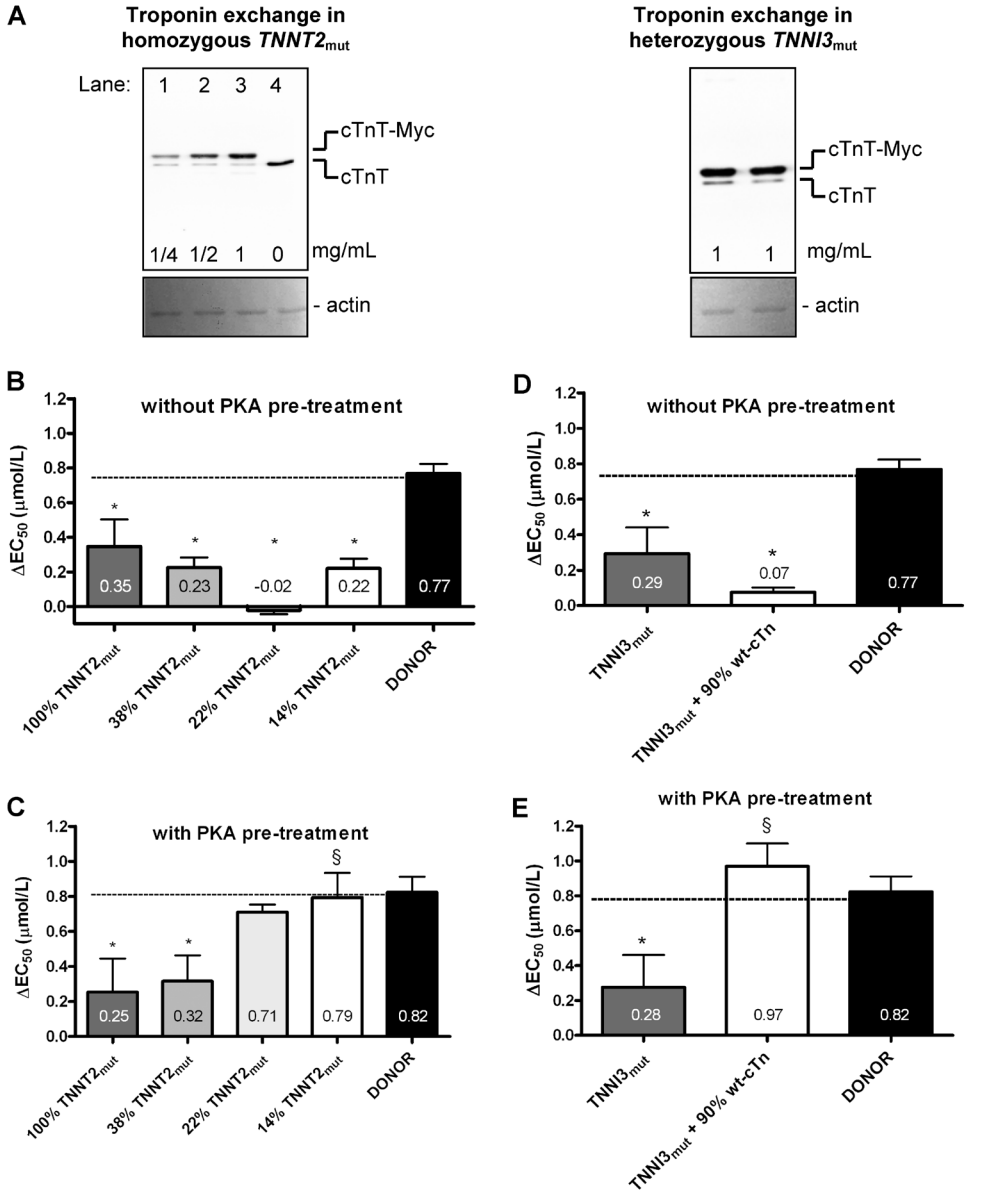

Figure 5. Replacement of endogenous mutant cardiac troponin by recombinant human wild-type troponin complex. A. Quantification of troponin exchange in cardiomyocytes from a *TNNT2*$_{mut}$ and *TNNI3*$_{mut}$ heart. Immunoblots stained with an antibody against cardiac troponin T (cTnT) that recognizes both endogenous cTnT (lower band) and recombinant myc-tag labeled cTnT (cTnT-myc; upper band). Left panel: An example is shown of a suspension of cardiomyocytes from a *TNNT2*$_{mut}$ heart exchanged with increasing concentrations of wild-type human recombinant troponin complex. Exchange with 0.25 mg/mL (lane 1), 0.5 mg/mL (lane 2) and 1 mg/mL (lane 3) troponin complex. *TNNT2*$_{mut}$ heart without added recombinant troponin complex (lane 4). Right panel: A suspension of cardiomyocytes from a *TNNI3*$_{mut}$ heart exchanged with 1 mg/mL wild-type human recombinant troponin complex. Similar

amounts were loaded on the blots (shown by Ponceau-stained actin) to allow cTnT analysis within the linear detection range. B. The length-dependent activation was significantly lower compared to donor in all $TNNT2_{mut}$ cells harboring varying amounts of mutant cTnT (100% without exchange and 38%, 22% and 14% in cells exchanged with increasing concentrations of unphosphorylated recombinant wild-type troponin). C. Measurements were also performed in exchanged cardiomyocytes, which were subsequently treated with PKA to normalize cTnI phosphorylation. Pre-treatment with exogenous PKA was not able to recover the blunted length-dependent activation in $TNNT2_{mut}$ cells with 38% of mutant cTnT, but did recover the reduced length-dependence of $TNNT2_{mut}$ exchanged with 78% and 86% wild-type troponin. D. The length-dependent activation was significantly lower in $TNNI3_{mut}$ cells exchanged with ~90% unphosphorylated recombinant wild-type troponin (wt-cTn) compared to donor cells. E. Pre-treatment with exogenous PKA restored the blunted length-dependent activation in $TNNI3_{mut}$ cells exchanged with wt-cTn complex to donor values. $^{*}p<0.05$ versus donor; $^{\S}p<0.05$ versus $TNNT2_{mut}$ or $TNNI3_{mut}$.

dependent activation. Our data indicate that mutant proteins resulting from missense mutations could perturb length-dependent sarcomere activation and underlie cardiac dysfunction observed at early stages of HCM disease development.

Myofilament Ca^{2+}-sensitivity and phosphorylation background

Our studies in human HCM with mutations in both thick and thin filament proteins showed high myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm (Figure 1A). This is in agreement with previous studies in transgenic mouse models and *in vitro* studies with mutant proteins, which indicate that HCM sarcomere mutations sensitize myofilaments to calcium.[3, 12, 13, 28] However, the higher Ca^{2+}-sensitivity in HCM_{mut} groups compared to non-failing donors coincided with lower phosphorylation levels of cMyBP-C and cTnI (Figure 3A). The difference in myofilament Ca^{2+}-sensitivity between HCM_{mut} and non-failing donor can thus be partly explained by hypophosphorylation of sarcomeric proteins rather than the sarcomere gene mutation itself.

A possible explanation for the low phosphorylation levels may reside in a blunted β-adrenergic response in HCM patients. A blunted response to isoproterenol, a β-adrenoreceptor agonist, has been reported in transgenic mice harboring *TPM1* and *MYH7* mutations.[29, 30] Moreover, reduced β-adrenoreceptor density has been reported in HCM patients.[31] Treatment of HCM samples with PKA increased phosphorylation of the PKA-target proteins (Figures 3 and 4 and Online Figure II) and normalized myofilament Ca^{2+}-sensitivity in $MYBPC3_{mut}$, $TPM1_{mut}$ and HCM_{smn} to values observed in non-failing donor. In contrast, after PKA higher myofilament Ca^{2+}-sensitivity was still present in $MYH7_{mut}$ and $TNNT2_{mut}$, suggesting a Ca^{2+}-sensitizing effect by these mutations. Interestingly, PKA-treatment significantly lowered Ca^{2+}-sensitivity of the $TNNI3_{mut}$ R145W compared to donor. This observation contrasts with transgenic animal models and reconstituted thin-filaments using recombinant human mutant cTnI.[32-34] It has been suggested that the Ca^{2+}-sensitizing action of mutations in the inhibitory region of cTnI (residues 137-148) directly impair the intrinsic inhibitory activity of cTnI.[32, 33] A possible explanation for the contradicting

results compared to our *TNNI3*$_{mut}$ samples may reside in the amount of endogenous mutant protein expression. Using adenovirus gene transfection to incorporate the cTnI R145W mutant into adult rat cardiomyocytes, Davis et al.[35] observed no elevation of myofilament Ca^{2+}-sensitivity, which was attributed to poor incorporation (~35%) of mutant protein into the sarcomeric structure compared with wild-type cTnI. In addition, the phosphorylation background of the sarcomeres may have been different among studies.

Overall, our data show that high Ca^{2+}-sensitivity is not a specific characteristic of human sarcomere mutation-positive HCM, as a similarly high myofilament Ca^{2+}-sensitivity was found in HCM$_{smn}$ compared to donors, which may be ascribed to reduced β-adrenergic signaling as part of cardiomyopathy development. Although PKA is the archetypical kinase involved in modulating Ca^{2+}-sensitivity through cTnI and cMyBP-C phosphorylation, it is by no means the only kinase that phosphorylates myofilament proteins. Both cTnI and cMyBP-C are target for a whole range of kinases.[36-40] cMyBP-C phosphorylation is thought to mainly affect cross-bridge cycling kinetics,[41] although a role in mediating Ca^{2+}-sensitivity of force has been suggested.[42] Cardiac TnI is considered to be the key regulator of Ca^{2+}-sensitivity, and it is mainly through phosphorylation of Ser23 and Ser24 that PKA exerts its Ca^{2+}-desensitizing effect, though many other phosphorylation sites have been identified.[43] Phosphorylation is but one of many possible post-translational modifications (PTMs). Recent reports have hinted at possible involvement of other PTMs in the regulation of sarcomere function, such as oxidation and S-glutathionylation[44] or O-GlcNAcylation.[45] It would thus be an oversimplification to propose that a reduction in PKA-phosphorylation of sarcomere proteins is solely responsible for the myofilament changes in HCM. However, the baseline Ca^{2+}-sensitivity seems to be dominated by the relatively low phosphorylation levels of PKA myofilament target proteins compared to donors. Higher phosphorylation levels mimicked by exogenous PKA treatment, as would be induced during increased cardiac stress (e.g. exercise), unveils a higher, similar or even lower myofilament Ca^{2+}-sensitivity in HCM dependent on the affected gene. Our findings suggest diverse mutation-induced changes in Ca^{2+}-sensitivity, while high myofilament Ca^{2+}-sensitivity is partly explained by secondary disease-related changes in protein phosphorylation.

Impairment of length-dependent sarcomere activation in HCM

Our data indicate that mutant sarcomeric proteins in HCM perturb length-dependent activation of myofilaments, which may contribute to early cardiac dysfunction observed in sarcomere mutation carriers. Studies in transgenic mouse models and troponin-exchange techniques with mouse tissue harboring HCM mutations were not conclusive as a reduced or normal length-dependent activation was found compared to controls.[30, 46-49] The recent study of Ford et al.[49] may shed some light on these previous observations as the authors studied length-dependent differences in mice expressing *TNNT2* mutations either in a α-MyHC (predominant in murines) or β-MyHC

(predominant in healthy adult human hearts) background. It was observed that mice expressing the R92L mutation in the physiological α-MyHC background presented a normal length-dependent Ca^{2+}-activation, while in the presence of the slow cycling β-MyHC isoform, length-dependence was lost.[49] Defects in length-dependent sarcomere properties were not similar in all HCM samples, as the blunted length-dependent increase in myofilament Ca^{2+}-sensitivity was corrected to donor values by exogenous PKA in truncating $MYBPC3_{mut}$ and HCM_{smn}, while it remained defective in HCM with missense mutations in *MYBPC3, MYH7*, *TNNT2*, *TNNI3* and *TPM1*. Moreover, the increase in maximal force generating capacity upon an increase in sarcomere length was almost entirely absent in HCM with troponin mutations (Table 2). Although PKA increased phosphorylation of cTnI and cMyBP-C and reduced myofilament Ca^{2+}-sensitivity at 2.2 μm in all HCM samples (except in the homozygous $TNNT2_{mut}$), it did not restore length-dependent activation in HCM samples with missense mutations. Intriguingly, a negative myofilament length-dependent activation was observed after PKA in the $TPM1_{mut}$ sample (Figure 2D), suggesting that length-dependent activation is even more impaired during increased cardiac stress.

The blunted length-dependent activation may be partly related to the relatively low phosphorylation of PKA-targets and high baseline myofilament Ca^{2+}-sensitivity. The suggestion that PKA-mediated myofilament protein phosphorylation has a modulatory role in length-dependent activation comes from studies in ferret papillary muscles,[26] in which isoprenaline, a stimulator of the β-adrenergic receptor pathway, enhanced the length-dependent change in the force-Ca^{2+} relation. Studies in cardiac tissue in which cTnI was replaced by skeletal TnI (ssTnI), which lacks the PKA-target serines (Ser23/24), showed higher myofilament Ca^{2+}-sensitivity, but a significantly reduced length-dependent activation,[27, 50] indicating a role for cTnI phosphorylation in length-dependent activation. A study by Cazorla et al.[47] in transgenic mice lacking cMyBP-C demonstrated lower length-dependent activation than wild-type mice that could not be restored by exogenous PKA treatment, which suggests that cMyBP-C is needed for proper length-dependent sarcomere activation. In our human samples with truncating *MYBPC3* mutations impaired length-dependent activation was corrected to donor values by PKA. Our previous study in HCM with truncating mutations in *MYBPC3* showed reduced expression of full-length cMyBP-C to ~70% compared to donor values (i.e. haploinsufficiency). Overall, our studies in human HCM with *MYBPC3* truncation mutations indicate that the presence of ~70% of full-length cMyBP-C protein in the sarcomere is sufficient to preserve the length-dependent properties of the sarcomeres.[15]

The perturbations in length-dependent activation in HCM with mutations in thin filament genes may be explained by the three-state model of filament transition, in which the troponin-tropomyosin complex has a central regulatory role. Myofilament contraction and force production is tightly regulated by the troponin-tropomyosin complex that regulates the interaction between the actin and myosin filaments. It is believed that the myofilaments exist in a dynamic equilibrium between three

biochemical transitions (Figure 6) that reflect different interactions between actin and myosin, termed the blocked (B-state), closed (C-state) and open (M-state) states of thin filament regulation.[51, 52] In the B-state Ca^{2+} is not bound to cTnC and tropomyosin sterically blocks myosin-binding sites on F-actin (Figure 6A). In the C-state Ca^{2+} binds to cTnC, which changes conformation of the troponin-tropomyosin complex, resulting in non-tension-generating cross-bridges which bind weakly to F-actin (i.e. weakly-bound cross-bridges) (Figure 6B).[52-54] The M-state, involves strong-binding of tension-generating cross-bridges which results in myofilament contraction and force development (Figure 6C).[52-54] Because of the central roles of cTnT and cTnI in the transition from the B-state to the C-state[55-57], it is likely that mutation-induced irregularities in protein interactions may translate into thin filament abnormalities.

The C-terminal half of cTnI docks the troponin-tropomyosin complex onto the outer domain of F-actin at low cytoplasmic [Ca^{2+}][58], stabilizing the formation of the B-state. Interestingly, ~86% of cTnI HCM-causing mutations (dashed red circle in Figure 6D), including the one present in our study, are found in the C-terminal half of cTnI (residues 137-210), a region responsible for actin-binding.[28, 59] Indeed, disruption of the B-state has been suggested in HCM-causing mutations affecting cTnI[60], but also cTnT.[61, 62] The relevance of the transition from the B- to the C-state for proper length-dependent activation has been shown by Smith and Fuchs[63] who were the first to provide evidence for a length-sensitive step in the transitions of thin filament activation. A reduction in ionic strength (<0.05 M), known to shift the B-state equilibrium towards a stable C-state,[64] coincided with impairment of length-dependent activation.[63] The blunted length-dependent increase in myofilament Ca^{2+}-sensitivity observed in the thin filament mutation groups can thus be explained by disruption of the B-state and an increased number of weakly-bound cross-bridges in the C-state.

Eleven HCM mutations have been identified in α-tropomyosin.[59] To the best of our knowledge the present study is the first to analyze the effects of a HCM-causing mutation (I284V) in the overlap region of tropomyosin, which has a central role in cooperative activation of the thin filament.[65, 66] The steepness of the force-Ca^{2+} relation, which is an indicator of the relative number of near-neighbor interacting sites, was significantly lower in $TPM1_{mut}$ compared to donors (1.98±0.37 and 3.33±0.11, respectively; Figure 2B), indicative for impairment of the cooperative response in activating the thin filament. Palm et al.[67] demonstrated that tropomyosin overlap regions are required for proper formation of a ternary complex with the N-terminal tail of cTnT. Since the N-terminal region of cTnT is needed to maintain the thin filament in the B-state,[56, 57] it is likely that mutations in the overlap region of α-tropomyosin structurally impair formation of the B-state and thereby impair length-dependent activation of myofilaments.

Previous studies indicated that myosin is not involved in the formation of the first two equilibrium states, i.e. B- and C-states.[52-54] However, myosin is crucial for formation of the M-state (myosin-induced), since strong-binding of tension-generating cross-

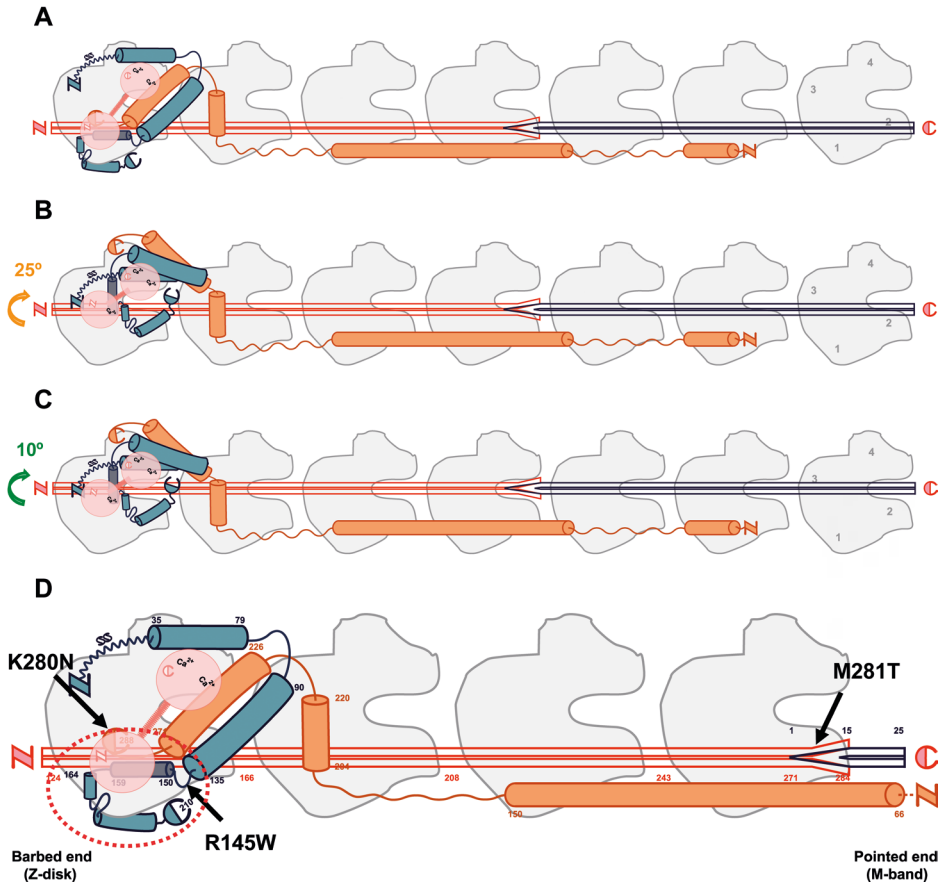

Figure 6. Schematic drawing of the thin filament functional unit. Seven actin monomers (gray) spanned by one tropomyosin dimmer (red) and one troponin complex: cTnC (pink), cTnI (blue) and cTnT (orange). Capitals "N" and "C" depict N- and C-terminal protein ends, respectively. Dark-blue tropomyosin depicts near-neighbor tropomyosin dimmer interaction.[70, 71] The orientation of thin filament proteins is as follows: the N-terminal region of cTnT points towards the pointed end (M-band), while the core domain of the troponin complex is oriented to the barbed end (Z-disk).[72] Interacting sites and structural location of actin-tropomyosin-troponin proteins are matched in accordance with available literature.[28, 53, 54, 58, 71, 73] Cardiac TnI residues 1-34 are arbitrarily positioned. Our drawing follows the proposed mechanism for Ca^{2+}-regulation of contraction proposed by Murakami et al.[58] **A.** B-state (blocked); when ATP is present and cytoplasmic [Ca^{2+}] is low such that Ca^{2+} is not bound to cTnC, tropomyosin sterically blocks the myosin-binding sites on F-actin. **B.** C-state (Ca^{2+}-induced); cytoplasmic [Ca^{2+}] rises such that Ca^{2+} binds to cTnC inducing conformational changes of the troponin complex, resulting in a ~25° movement of tropomyosin on the thin filament, thereby exposing most of the myosin-binding sites on F-actin. Note the movement of tropomyosin away from subdomains 1 and 2 of F-actin. In the C-state the myofilament is not yet activated as non-tension-generating cross-bridges bind weakly to F-actin. **C.** M-state (myosin-induced); involves the strong-binding of tension-generating cross-bridges that induce an additional ~10° movement of tropomyosin on F-actin, resulting in myofilament activation and contraction. Note the transition of tropomyosin into subdomains 3 and 4 of F-actin. **D.** Solid arrows depict the location of mutations' sites on thin filament proteins present in our human HCM samples. Cardiac TnT residues 1-65 are shortened to fit the enlarged scale.

bridges are required for thin filament activation and force production.[52-54] Since five of the six samples used in our study have mutations in the myosin S1 domain, responsible for actin-binding[68] it is likely that the deleterious effects of *MYH7* mutations occur via perturbation of the M-state. A recent study by Farman et al.[69] highlights the essential role of myosin heads' orientation that precede thin filament activation for proper lattice spacing and length-dependent activation.

Thus altered myosin head orientation as a result of mutations may impair formation of the M-state and affect length-dependent activation.

The possible involvement of cMyBP-C in the modulation of thin-filament activity has been only recently addressed. Electron microscopy and 3D reconstruction of thin-filaments with cMyBP-C suggest that the N-terminal extension of cMyBP-C could modulate the movement of tropomyosin on F-actin and interfere with actomyosin interactions, possibly involved in the regulation of thin-filament activation.[74] The *MYBPC3* missense mutations in our study are located along the N-terminal extension of cMyBP-C and may alter the tropomyosin-actin interaction and thereby impair length-dependent activation.

Study limitations and clinical implications

Although we were able to study a large collection of human HCM samples with different mutations in thick and thin filament proteins, care must be taken to extrapolate our findings to all HCM patients. Our HCM population consisted of patients with LV outflow tract obstruction, and the patient with the homozygous *TNNT2* mutation had end-stage heart failure. Our data do highlight that mutation-induced changes in myofilament Ca^{2+}-sensitivity and length-dependent sarcomere activation are diverse and depend on the affected gene, and most likely location and type of the mutation in the affected protein. We provide evidence that mutant protein may impair sarcomere function at ~38% expression (Figure 5C), which emphasizes the importance of studying the mutant protein level at which cardiac performance is impaired. Future studies in transgenic mice models and human myectomy samples are warranted to extend our findings to a broader set of sarcomere mutations and assess the toxic dose of mutant proteins.

Although sarcomere mutation-negative patients are commonly used as control group, we cannot completely rule out the presence of rare mutations. However, as our cardiomyocyte analyses revealed no functional impairments (PKA normalized length-dependent activation in HCM_{smn}), the likelihood of the presence of a rare mutation is low.

Our study revealed perturbed sarcomere length-dependent activation as a common mechanism underlying cardiac dysfunction in HCM. Pathological hypertrophy presumably reflects the compensatory response of the heart to counteract impaired sarcomere defects such as the blunted length-dependent myofilament activation. The relatively low force generating capacity of cardiomyocytes and the inability to increase force upon an increase in sarcomere length may in part underlie

cardiac dysfunction and initiate compensatory hypertrophy. Pak and colleagues[75] showed a blunted end-systolic pressure volume relation in HCM patients, suggesting that the hearts were unable to properly recruit preload to augment contractility. The latter observation may be explained by cardiac remodeling. However, likewise, the mutation-induced blunted length-dependent sarcomere activation may limit preload-mediated contractile reserve of the heart in HCM patients. Our study in combination with others[6-10] supports the hypothesis that defective sarcomere function as a result of gene mutations is central to early stages of HCM-disease and precedes development of hypertrophy. Moreover, the blunted increase in myofilament Ca^{2+}-sensitivity upon an increase in length may alter Ca^{2+}-buffering in the cardiomyocytes and provide a substrate for arrhythmias.

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Disclosures

None

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Editorial

Familial Hypertrophic Cardiomyopathy

Is the Frank–Starling Law Kaput?

Sabine Huke, Björn C. Knollmann

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Editorial

Familial Hypertrophic Cardiomyopathy
Is the Frank–Starling Law Kaput?

Sabine Huke, Björn C. Knollmann

Building on work by Otto Frank in the late 19th century, Ernest Starling described in the early 20th century the ability of the heart to change its force of contraction and therefore stroke volume in response to changes in venous return, which has been called the Frank–Starling law of the heart in honor of these 2 physiologists. The Frank–Starling effect enables the heart to match cardiac output to venous return on a beat-to-beat basis.[1] A major mechanism responsible for the Frank–Starling effect and hence the beat-to-beat autoregulation of cardiac output is the sarcomere length-dependent activation of force development.[2]

Article, see p 1491

In this issue of *Circulation Research*, Sequeira et al[3] investigate both the Ca- and length-dependent regulation of force development in cardiomyocytes harvested from patients with hypertrophic cardiomyopathy (HCM), a familial disorder commonly associated with mutations in genes encoding sarcomeric proteins.[4] Consistent with previous animal and human studies,[5] myofilament Ca sensitivity was increased in all 38 HCM patient samples compared with 12 nonfailing donor hearts. Notable, the authors observed that length-dependent activation was impaired, which, unlike the Ca sensitivity increase, seemed to be a primary effect of the mutant sarcomeric proteins because (1) it could not be rescued by increasing protein kinase A (PKA) phosphorylation and (2) was normalized when mutant troponin (Tn) proteins were replaced with wild-type protein. These new results suggest that impaired length-dependent activation and hence a defective Frank–Starling effect importantly contribute to the pathogenesis of HCM (Figure).

Reduced Maximal Ca-Activated Force and Increased Ca Sensitivity Are Central Findings in Human HCM

HCM is a familial disorder commonly associated with mutations in genes encoding sarcomeric proteins. Mutation carriers develop progressive heart hypertrophy and fibrosis and are at a high risk for sudden death because of ventricular arrhythmia. More than 1000 mutations in >11 genes have been identified, which can lead to this disease. The current report,[3] together with 2 previous smaller human studies,[6,7] indicates that, regardless of the disease-causing mutation, human HCM cardiac muscle exhibits reduced maximal Ca-activated force and increased Ca sensitivity of muscle activation. This result raises the possibility that HCM mutations trigger a common cascade of molecular events that result in a similar phenotype (Figure).

Based largely on in vitro studies and animal models, myofilament Ca sensitization had previously been associated with HCM.[8,9] The study by Sequeira et al[3] suggests that myofilament Ca sensitization indeed is a universal finding in human HCM. However, the authors find that Ca sensitization is often not a primary property conferred by the mutant protein itself. Rather, the increased Ca sensitivity was largely a result of an apparent hypophosphorylation of PKA targets in the myofilaments. Although all HCM groups had higher myofilament Ca sensitivity at baseline, in tissue from mutation carriers in myosin-binding protein C, TnI, and tropomyosin and from HCM patients without sarcomeric mutations, Ca sensitivity was restored to normal levels by treatment with PKA. The 2 exceptions were one patient with a mutation in TnT and another patient with a β-myosin heavy chain mutation, where the Ca sensitivity increase seemed to be a property directly conferred by the mutant protein. As with any patient study, differences in drug therapy between the HCM and the normal donor group may have contributed to reduced PKA phosphorylation. Nevertheless, this result suggests that increased Ca sensitivity of human HCM muscle is mostly the result of posttranslational modifications and potentially helps to maintain systolic function, despite the reduction in maximal Ca-activated force caused by HCM mutations.[7] Regardless of its underlying cause, the Ca sensitivity increase is likely of major importance for HCM pathophysiology, with experimental evidence indicating that, regardless of how myofilaments are Ca sensitized, an increase in myofilament Ca sensitivity can contribute to diastolic dysfunction,[10] focal energy deficits,[11] and arrhythmogenesis[12] (Figure).

Impaired Length-Dependent Activation: A Trigger of HCM Pathogenesis?

The authors discovered a new common finding in human HCM muscle—impaired length-dependent activation, which, in contrast to increased Ca sensitivity, was not rescued by PKA treatment for all sarcomeric missense mutations studied. Furthermore, replacement of mutant with wild-type Tn corrected the impaired length dependence in the 2 mutations that could be studied. Hence, impaired length-dependent activation

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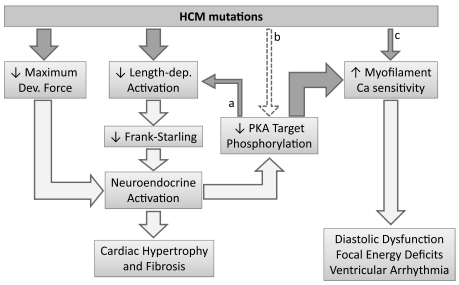

Figure. Pathophysiological framework of hypertrophic cardiomyopathy (HCM). Solid arrows indicate experimental findings reported by Sequeira et al.[3] Open arrows indicate the consequences based on experimental evidence from other studies. (a) Impaired length-dependent activation can also be caused by protein kinase A (PKA) hypophosphorylation (ie, of troponin I [TnI]). (b) HCM mutations may directly interfere with phosphorylation of myofilament PKA targets. (c) Only a subset of HCM mutations directly alter myofilament Ca sensitivity.

may be a direct effect of many mutations that cause HCM in humans (Figure). What is the significance of this finding for the pathogenesis of HCM? Because a functional length-dependent activation is responsible for the Frank–Starling effect, it is intriguing to speculate that HCM mutations generate an intermittent inability to match cardiac ejection to dynamic changes in ventricular filling volume (ie, during the respiratory cycle or during exercise). The resulting increase in wall stress as a result of intermittent impaired ventricular emptying could trigger neuroendocrine activation and a hypertrophic gene program analogous to acquired heart disease with increased wall stress and an impaired Frank–Starling effect, such as postmyocardial infarction cardiomyopathy. Although not tested directly in the report by Sequeira, activation of neuroendocrine signaling could be the main culprit for the decrease in PKA phosphorylation of myofilament target proteins found universally in patients with HCM (Figure). In addition, some of missense HCM mutation (ie, in TnT or TnI) may directly interfere with the ability of PKA to phosphorylate TnI (Figure, dashed arrow b). PKA phosphorylation of TnI not only regulates myofilament Ca sensitivity but also is important for physiological length-dependent activation of cardiac muscle.[13] Hence, PKA hypophosphorylation can independently contribute to impaired length-dependent activation, as was demonstrated by the authors for 2 groups of patients with HCM (ie, truncation mutations of myosin-binding protein C and genotype-negative HCM). In those patients, this can generate a vicious cycle that could be central to the pathogenesis in HCM, as shown in the Figure.

In addition to the impaired length-dependent activation, which is measured experimentally by the increase in Ca sensitivity (ΔpCa50) induced by increased sarcomere length (Sequeira et al,[3] Figure 2), all sarcomeric missense mutations (but not myosin-binding protein C truncation mutants and genotype-negative HCM) also impaired the length-dependent increase in maximum force (Sequeira et al,[3] Table 2). The latter is measured at fully activating [Ca] and reflects the intrinsic force-generating ability of the muscle. Although there can be many reasons for the reduced length dependence of maximum force (eg, loss of myofibrils), the net result is an even further compromised length–tension relationship and hence impaired Frank–Starling effect.

Open Questions

Several questions remain. Given that genetic background might affect the functional effect of the mutations and many of the individuals studied by Sequeira et al[3] were family members, the results of the study may not be quite generalizable to the general HCM population. By necessity, all human myocardial samples came from patients with significant cardiac hypertrophy and heart failure. Hence, it is still possible that altered length-dependent activation is the consequence of pathological remodeling of the sarcomeric apparatus in the diseased myocardium. Nevertheless, the rescue with wild-type Tn protein replacement provides compelling evidence that this is not the case, at least for 2 specific mutations. Furthermore, passive viscoelastic properties of human HCM cardiomyocytes are similar to those of cardiomyocytes from healthy donor hearts,[7] further supporting the hypothesis that altered Ca and length-dependent regulation of muscle activation are central to the pathophysiology of human HCM (Figure). Not directly investigated in the current study, but likely also important for HCM pathophysiology is the inefficient ATP utilization (increased tension cost) caused by many HCM mutations.[14] It remains to be seen which of these myofilament properties is the most significant driver of the disease.

Altered myofilament properties affect every myocyte in the heart starting at birth, and although some heterogeneity is expected, there are significant questions remaining. For example, what is the reason for the incomplete penetrance of the disease (age dependent, but possibly as low as 41%)?[15] What is responsible for the focal nature of hypertrophy and fibrosis in HCM hearts? This clearly shows that the presence of an HCM mutation does not inevitably lead to disease, and even in an affected patient some regions of the heart remain normal. Such a phenotype may imply a second hit that is necessary to initiate the disease and remains to be identified. A potential culprit is the focal energy deprivation that occurs in HCM mouse models during stress,[11] which can directly contribute to reentry arrhythmias[11] but also has the potential to drive heterogeneous hypertrophy and remodeling.[16] The next frontier will be to test whether therapeutic interventions aimed at normalizing Ca sensitivity, length-dependent activation, or inefficient ATP utilization are beneficial in HCM.

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Disclosures

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KEY WORDS: Editorials ■ Frank–Starling law ■ human cardiomyocytes ■ length-dependent activation

4

LENGTH-DEPENDENT ACTIVATION IS MODULATED BY CARDIAC TROPONIN I BISPHOSPHORYLATION AT SER23 AND SER24 BUT NOT BY THR143 PHOSPHORYLATION

Wijnker PJM, Sequeira V, Foster DB, Li Y, dos Remedios C, Murphy AM, Stienen GJM, van der Velden J

American Journal of Physiology 2014; 306:H1171-H1181

The greatest discovery during my PhD? The Higgs boson at CERN, July 4, 2012

Vasco Sequeira

ABSTRACT

Frank-Starling's law reflects the ability of the heart to adjust the force of its contraction to changes in ventricular filling, a property based on length-dependent myofilament activation (LDA). The threonine at amino acid 143 of cardiac troponin I (cTnI) is prerequisite for the length-dependent increase in Ca^{2+}-sensitivity. Thr143 is a known target of protein kinase C (PKC) whose activity is increased in cardiac disease. Thr143 phosphorylation may modulate length-dependent myofilament activation in failing hearts. Therefore, we investigated if pseudo-phosphorylation at Thr143 modulates length-dependence of force using troponin exchange experiments in human cardiomyocytes. In addition, we studied effects of protein kinase A (PKA)-mediated cTnI phosphorylation at Ser23/24, which has been reported to modulate LDA.

Isometric force was measured at various Ca^{2+}-concentrations in membrane-permeabilized cardiomyocytes exchanged with recombinant wild-type (Wt) troponin or troponin mutated at the PKC site Thr143, or Ser23/24 into aspartic acid (D) or alanine (A) to mimic phosphorylation and dephosphorylation, respectively. In troponin-exchanged donor cardiomyocytes experiments were repeated after incubation with exogenous PKA.

Pseudo-phosphorylation of Thr143 increased myofilament Ca^{2+}-sensitivity compared to Wt without affecting LDA in failing and donor cardiomyocytes. Subsequent PKA treatment enhanced the length-dependent shift in Ca^{2+}-sensitivity after Wt and 143D exchange. Exchange with Ser23/24 variants demonstrated that pseudo-phosphorylation of both Ser23 and Ser24 is needed to enhance the length-dependent increase in Ca^{2+}-sensitivity. cTnI pseudo-phosphorylation did not alter length-dependent changes in maximal force.

Thus phosphorylation at Thr143 enhances myofilament Ca^{2+}-sensitivity without affecting LDA, while Ser23/24 bisphosphorylation is needed to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity.

Key Words: myofilament function, protein phosphorylation, troponin I

INTRODUCTION

Frank-Starling's law of the heart describes the ability of the heart to adjust the force of its contraction (stroke volume) to changes in ventricular filling (end-diastolic volume), a property based on length-dependent activation (LDA) of the myofilaments (7, 8). An increase in sarcomere length within the working range of the heart enhances the maximal force generating capacity and the sensitivity of the myofilaments to calcium. Length-dependent myofilament activation thus represents an important cellular mechanism to adjust cardiac performance in response to increased preload of the heart.

Cardiac troponin I (cTnI) is an important regulator of LDA of the myofilaments. This has been elegantly demonstrated via replacement of endogenous cTnI by slow skeletal troponin I (ssTnI) in cardiac muscle, which significantly reduced the increase in myofilament Ca^{2+}-sensitivity upon an increase in sarcomere length (2, 16, 33). More recently, it was demonstrated that the threonine residue 143 (Thr144 in rat and mice, Thr143 in human) of the inhibitory region of cTnI is essential for length-dependent alterations in myofilament Ca^{2+}-sensitivity. Substitution of the threonine for a proline at 143 ($cTnI_{Thr143Pro}$), as present in ssTnI and fast skeletal TnI, largely ablated the length-dependent increase in myofilament Ca^{2+}-sensitivity in cardiac muscle (33).

Interestingly, Thr143 is a well-known target of protein kinase C (PKC) and its phosphorylation has been implicated in heart failure (25, 32, 43) where an increase in PKC-mediated cTnI phosphorylation has been found together with a decreased phosphorylation of the protein kinase A (PKA) sites (43). PKC-mediated Thr143 phosphoryation may, either alone or in concert with down-regulated PKA-mediated phosphorylation, underlie changes in LDA in the heart. In the present study we therefore first investigated the effect of Thr143 phosphorylation on LDA in human failing cardiomyocytes. Troponin exchange experiments were used to study the functional effects of pseudo-phosphorylated cTnI at Thr143 (mimicked by aspartic acid) in comparison to unphosphorylated (mimicked by alanine) and wild-type cTnI.

The effect of Thr143 phosphorylation may depend on the phosphorylation status of Ser23/24, since it has been demonstrated that PKA-mediated cTnI phosphorylation enhances the length-dependent increase in myofilament Ca^{2+}-sensitivity (16). Therefore, secondary experiments were performed with and without exogenous PKA incubation after cTn exchange in non-failing donor cardiomyocytes. Also, as previous experiments showed that PKA-bisphosphorylation at Ser23 and Ser24 is required to decrease Ca^{2+}-sensitivity (39, 44) we investigated effects of pseudo-mono- and pseudo-bisphosphorylation at cTnI-Ser23/24. To study the effect of Ser23/24 phosphorylation on LDA, experiments were performed in failing cardiomyocytes that have a low baseline level of cTnI-Ser23/24 phosphorylation.

Our data show that pseudo-phosphorylation of Thr143 in human cardiomyocytes increases myofilament Ca^{2+}-sensitivity without affecting LDA of the sarcomeres. Moreover, in human myocardium Ser23/24 bisphosphorylation is required to increase the length-dependent shift in myofilament Ca^{2+}-sensitivity.

MATERIALS AND METHODS

Human myocardial tissue

Troponin exchange experiments were performed in cardiomyocytes from end-stage failing idiopathic dilated cardiomyopathy (IDCM) hearts (2 males/1 female, left ventricular ejection fraction 16.7±4.4%, age 54.3±1.9 years) or from non-failing donor myocardium, obtained during heart transplantation surgery. The tissue was collected in cardioplegic solution and stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the Human Research Ethics Committee of The University of Sydney (#7326). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997). The human cardiac samples used were extensively characterized (cardiomyocyte force characteristics and cTnI phosphorylation) in a previous study (10).

Exchange of human troponin complex experiments

Expression and purification of recombinant troponin subunits

Recombinant human troponin complex was produced as described in detail previously (39). Shortly, six different cardiac troponin I (cTnI) forms were made via site-directed mutations of Thr143 and Ser23/Ser24 into aspartic acid (D) to mimic phosphorylation or into alanine (A) to mimic dephosphorylation: 143D, 143A, 23D/24D, 23D/24A, 23A/24D, 23A/24A. cDNA encoding human cardiac isoforms (troponin C (cTnC), myc-tag labeled cTnT (cTnT-myc), wild-type cTnI and cTnI mutants) were transformed in E. coli Rosetta2 (22) and cultured under carbenicillin/chloramphenicol selection in Overnight Express TB medium (EMD Biosciences). Cultures were harvested by centrifugation, re-suspended in PBS, and centrifuged at 10000xg. Pellets were stored at -80°C until use.

Troponin subunits were purified using fast protein liquid chromatography (AKTA-FPLC System; Amersham Biosciences) as described previously (22).

Reconstitution of troponin complexes

Fractions containing equal ratios of cTnT, cTnC and cTnI subunits were pooled, subsequently purified by AKTA-FPLC chromatography using a Resource Q to remove residual uncomplexed troponin subunits and finally dialyzed against 10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 0.1 mM PMSF (pH 6.9; 2 times, 1L each) prior to concentrating the complexes to a final concentration of >2 mg/mL by centrifugation using Centriprep YM-10 centrifugal filters (Millipore).

Exchange of human troponin complex

Exchange of recombinant cTn in human cardiomyocytes was done as described previously (39). Briefly, single cardiomyocytes were mechanically isolated with a glass tissue homogenizer, and permeabilized by Triton X-100 (0.5%; v/v) for 5 minutes. They were subsequently incubated overnight at 4°C in exchange solution containing

1 mg/mL of recombinant human cTn complex with the addition of 4 mM $CaCl_2$, 4 mM DTT, 5 ml/mL protease inhibitor cocktail (Sigma, P8340) and 10 ml/mL phosphatase inhibitor cocktail 2 and 3 (Sigma, P5726 and P0044) (pH 6.9). The next day, the cardiomyocytes were washed twice in rigor solution and finally in relaxing solution (5.95 mM Na_2ATP, 6.04 mM $MgCl_2$, 2 mM EGTA, 139.6 mM KCl, 10 mM imidazole, pH 7.0). This method results in a homogenous distribution of recombinant cTn complex within the exchanged cardiomyocytes (22).

Determination of the degree of troponin exchange

The degree of troponin exchange was determined as described previously (39). Briefly, to determine the degree of cTn exchange and to assess protein phosphorylation status, part of the suspension of cells was treated with 2D-clean-up kit (GE Healthcare) as described by the manufacturer protocol after overnight cTn exchange. Subsequently, tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT. Protein concentration measured with RCDC Protein Assay Kit II (BioRad) ranged between 2 to 4 mg/mL.

Immunoblotting was used to determine the degree of exchange of endogenous cTn by recombinant cTn complex. Therefore, recombinant cTnT was labeled with a Myc-tag to allow discrimination between endogenous and recombinant cTn complex. In a previous study we demonstrated that Myc-tag labeling does not interfere with myofilament function (39). Proteins were separated on a one-dimensional 13% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond) using the protocol supplied by the manufacturer in 1 hour at 75 V. A specific monoclonal antibody against cTnT (Clone JLT-12, Sigma; dilution 1:1250) was used to detect endogenous and recombinant cTnT by chemiluminescence (ECL, Amersham Biosciences). We have previously demonstrated that the affinity of the cTnT antibody was the same for cTnT compared to cTnT-Myc and that cTnT loading was within the linear range (39).

Myofilament protein phosphorylation

The phosphorylation levels of sarcomeric proteins was determined before and after cTn exchange using ProQ-Diamond stained 1D-gels, as described previously (39). The phosphorylation signals were normalized to the intensities of the SYPRO Ruby stained myosin light chain 2 bands to correct for small differences in protein loading. The PeppermintStick Phosphoprotein marker (Molecular Probes) was used to correct for differences in staining between gels (41). The ratio of the intensities of ProQ-Diamond and SYPRO Ruby stained ovalbumin band was used to correct for inter-gel differences.

The distribution of endogenous phosphorylated species of cTnI was analyzed using Phos-tag™ acrylamide gels (FMS Laboratory; Hiroshima University, Japan) as described before (10, 18).

Analysis of titin isoform composition

Tissue samples were weighed and pulverized in liquid nitrogen using a mortar and a pestle. Tissue powder was solubilized in 8M urea buffer with DTT and 50% glycerol solution with protease inhibitors (0.16 mmol/L Leupeptin, 0.04 mmol/L E-64 and 0.2 mmol/L PMSF). Samples were loaded in triplicate on 1% agarose gels stained with SYPRO Ruby to determine titin isoform composition as described previously (27).

Isometric force measurements

Force measurements in cardiomyocytes exchanged with recombinant cTn were performed as described previously (17, 37). Isometric force was measured at 15°C and LDA was determined by measuring force at different Ca^{2+} concentrations, first at a sarcomere length of 1.8 µm and subsequently at 2.2 µm. The following parameters were determined: passive force at pCa 9.0 (F_{pas}), maximal force at pCa 4.5 (F_{max}: total force minus F_{pas}), Ca^{2+}-sensitivity of force development (pCa_{50}) and steepness of the sigmoidal force-pCa relation (nHill). Only a minor decrease in maximal force development was observed during force measurements at a sarcomere length of 1.8 (2.1±2.5%) and 2.2 (7.8±1.9%) µm. Force decline during the entire experiment did not differ between cells exchanged with the different troponin complexes. Troponin-exchanged donor cardiomyocytes were incubated with exogenous PKA for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U/incubation, Sigma) after which isometric force measurements were performed at 15°C.

Data analysis

Data analysis was performed as previously described using the Hill equation to fit force-Ca^{2+} relations: $F(Ca^{2+})/F_0=[Ca^{2+}]^{nHill}/(Ca_{50}{}^{nHill}+[Ca^{2+}]^{nHill})$

Where F is steady-state force, F_0 the steady-state force at saturating $[Ca^{2+}]$, nHill the steepness of the relationship and Ca_{50} (or pCa_{50}) represents the midpoint of the relation. One-way ANOVA followed by a Bonferroni post-hoc test was used to compare the amount of exchange of the different cTn-complexes. Two-way ANOVA repeated measures followed by a Bonferroni post-hoc test was used to compare groups exchanged with the different cTn complexes (#$P<0.05$, significant difference compared to control (Wt for Table 2-4; 23A/24A for Table 5)). When two-way ANOVA revealed a significant effect for sarcomere length ($P<0.05$), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*$P<0.05$, 1.8 vs 2.2 µm). Data values represent an average of all cells of the different IDCM hearts. No significant differences were found between the 3 IDCM hearts after cTn exchange with the same complex for all force parameters measured. Values are given as means ± S.E.M. of n myocytes.

RESULTS

Pseudo-phosphorylation of Thr143 increases Ca^{2+}-sensitivity without affecting length-dependent activation in IDCM cardiomyocytes

As the effect of pseudo-phosphorylated Thr143 may be modulated by cTnI phosphorylation at Ser23/24, we first analyzed baseline cTnI phosphorylation in the idiopathic dilated cardiomyopathy (IDCM) samples used in the exchange experiments. Phostag analysis showed a relatively low cTnI phosphorylation in IDCM hearts (n=3): 7.5 ± 0.9% bisphosphorylated, 27.2 ± 4.7% monophosphorylated and 65.3 ± 5.1% unphosphorylated cTnI (Figure 1A).

Troponin exchange was determined using immunoblot detection of the myc-tag labeled cTnT. Figure 1B depicts a representative immunoblot loaded with IDCM cardiomyocytes incubated overnight with recombinant cTn containing pseudo-dephosphorylated Thr143 (143A), pseudo-phosphorylated Thr143 (143D) and wild-type (Wt) cTnI. Myc-tagged cTnT migrates more slowly through the gel in comparison to endogenous cTnT and therefore 2 cTnT bands are found. The percentage of cTn exchange was calculated from the ratio of myc-tagged recombinant cTnT and the total amount of cTnT. The average exchange percentage of the 3 cTn complexes

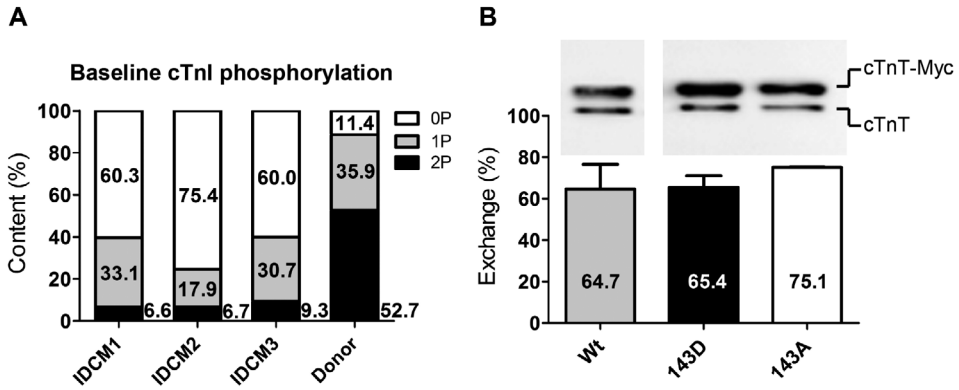

Figure 1. Baseline cardiac troponin I phosphorylation and quantification of troponin exchange in cardiomyocytes. A: Distribution of cardiac troponin I (cTnI) phosphospecies of the three IDCM samples and the donor sample used in the exchange experiments determined via a Phos tag-blot. B: Immunoblot stained with an antibody against troponin T (cTnT) that recognizes both endogenous cTnT (lower band) and recombinant Myc-tag-labeled cTnT (cTnT-myc). An example is shown of a suspension of IDCM cardiomyocytes exchanged with recombinant cTn (143A, 143D or Wt). The bar graphs indicate average percentages of cTn exchange in cardiomyocytes after overnight incubation in exchange solution containing the different cTnI species (average values from 3 IDCM hearts). No significant differences were found in exchange percentage between the three complexes. Error bars are visible when larger than symbol size. Abbreviations: 143A, pseudo-dephosphorylated cTnI at Thr143; 143D, pseudo-phosphorylated cTnI at Thr143; Wt, wild-type cTnI.

in IDCM amounted to 68.4 ± 3% and did not significantly differ (p = 0.58) between the 3 cTn complexes (Figure 1B), indicating similar incorporation in the myofilaments.

Previous studies indicated that sarcomeric proteins, other than cTnI, may modify length-dependent activation (9, 19). To assess if cTn exchange affected phosphorylation of myofilament proteins other than troponin, ProQ-Diamond staining was performed before and after cTn exchange. In agreement with our previous study (39), no significant differences in protein phosphorylation were induced in the other contractile proteins studied (cardiac myosin-binding protein-C, myosin light chain 2 and desmin) upon cTn exchange (Table 1). Since endogenous cTnT phosphorylation level was low, no significant reduction in cTnT phosphorylation could be detected upon exchange with unphosphorylated whole troponin complex via ProQ-diamond staining. The level of cTnT phosphorylation did not differ between cells exchanged with the different cTn complexes (Table 1).

Table 1. Myofilament protein phosphorylation before and after troponin exchange.

	cMyBPc	Desmin	MLC2	cTnT
Before exchange	0.39±0.02	0.47±0.13	0.35±0.10	0.20±0.04
143A	0.37±0.02	0.48±0.14	0.36±0.10	0.15±0.01
143D	0.37±0.03	0.46±0.17	0.33±0.09	0.15±0.02
Wt	0.35±0.03	0.45±0.08	0.29±0.03	0.17±0.02

Values are means ± SEM of the ProQ/SYPRO intensity ratio. Shown is myofilament protein phosphorylation determined with ProQ-diamond staining before and after exchange in three IDCM samples. Troponin exchange did not affect myofilament protein phosphorylation (by one-way ANOVA comparing before exchange to the exchanged groups). cMyBP-C, cardiac myosin-binding protein-C; MLC2, myosin light chain 2; cTnT, cardiac troponin T; A, alanine substitution; D, aspartic acid substitution; Wt, wild-type.

To determine the effects of Thr143 phosphorylation on myofilament force development, force measurements were performed in failing cardiomyocytes exchanged with 143D. As controls, 143A was used, where alanine was used to mimick dephosphorylation at Thr143, and unphosphorylated Wt cTnI. Ca^{2+}-sensitivity of force development was measured at 2.2 µm sarcomere length in the cTn-exchanged IDCM cardiomyocytes (1 mg/mL recombinant cTn; 3 IDCM hearts, 11-13 cells in total). Myofilament Ca^{2+}-sensitivity was significantly increased in 143D, evident from the leftward shift of the force-pCa curve for 143D compared to Wt (Figure 2A), while 143A did not change Ca^{2+}-sensitivity compared to Wt. The midpoint of the force-pCa relation (pCa_{50}) was significantly higher in 143D-exchanged cells compared to both controls (Wt and 143A; Figure 2B).

To study the effect of Thr143 phosphorylation on length-dependent myofilament activation, force measurements were also performed at 1.8 µm (Figure 2C). The effect of sarcomere lengthening on myofilament Ca^{2+}-sensitivity is displayed as the ΔpCa_{50}, which is the difference in pCa_{50} at a sarcomere length of 1.8 µm (Figure 2C) and

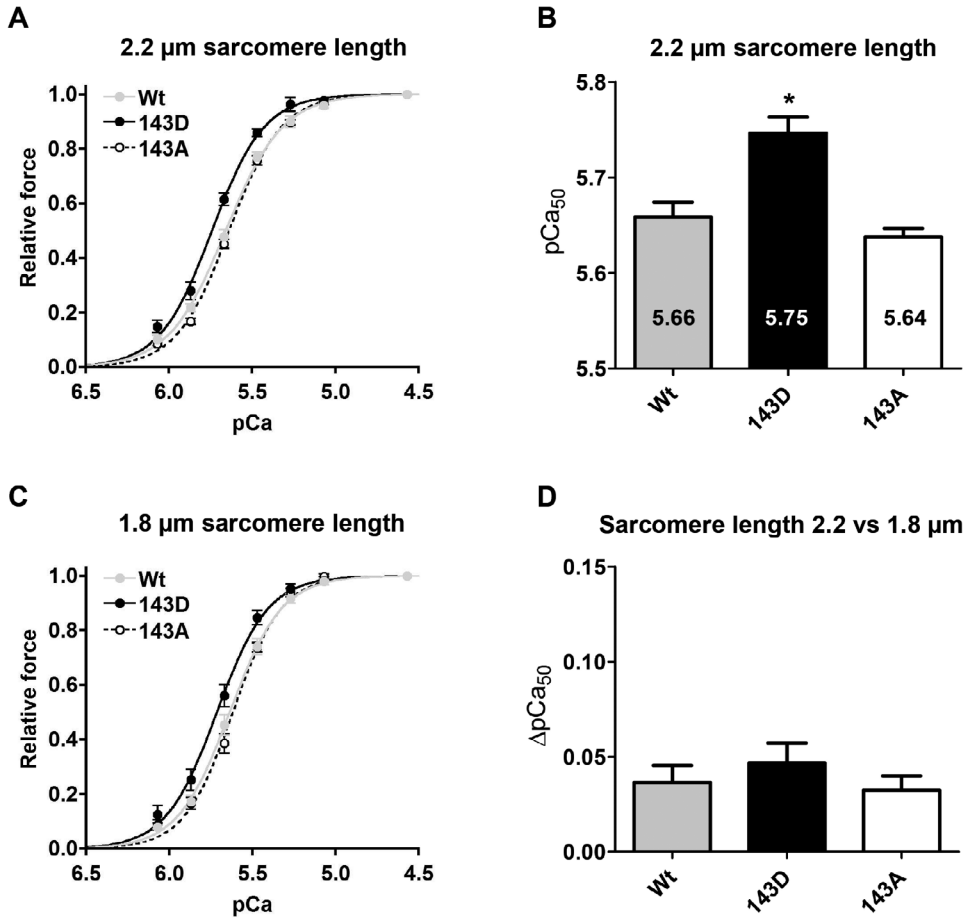

Figure 2. Myofilament Ca^{2+}-sensitivity is increased upon Thr143 pseudo-phosphorylation without affecting length-dependent activation in cardiomyocytes from failing human hearts. A: Myofilament force development measured at 2.2 µm sarcomere length at various [Ca^{2+}] in permeabilized cardiomyocytes from idiopathic dilated cardiomyopathy (IDCM) patients in which endogenous troponin complex was partially exchanged (68.4±3%) with recombinant myc-tag-labeled troponin complexes (11-13 cardiomyocytes in total from 3 IDCM hearts per group). B: Compared to unphosphorylated wild-type cTnI (Wt), Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) was significantly increased after exchange with pseudo-phosphorylated cTnI at Thr143 (143D). Pseudo-dephosphorylation at Thr143 (143A) did not alter Ca^{2+}-sensitivity compared to Wt. C: The length-dependent increase in Ca^{2+}-sensitivity was studied by measuring force at sarcomere lengths of 1.8 and 2.2 µm (8 cells for each group, 71.0±4.4% exchange). D: Exchange with 143D did not significantly alter the length-dependent increase in myofilament Ca^{2+}-sensitivity (ΔpCa_{50}: 1.8 versus 2.2 µm) compared to 143A and Wt. *P<0.05, 143D versus 143A and Wt in post-test Bonferroni analyses of one-way ANOVA.

2.2 µm (Figure 2A). For all cTn complexes, Ca^{2+}-sensitivity significantly increased upon cardiomyocyte lengthening. The increase in pCa_{50} did not differ among groups

(ΔpCa_{50} Wt; 0.04 ± 0.01, 143D; 0.05 ± 0.01, 143A; 0.03 ± 0.01; Figure 2D). Sarcomere lengthening from 1.8 to 2.2 µm significantly increased maximal (F_{max}) and passive (F_{pas}) force in all 3 cTn-exchanged groups (Table 2), which is in accordance with the well-known length-force relationship (1). nHill slightly decreased upon sarcomere lengthening, only reaching a significant difference for 143A (Table 2). No significant differences in F_{max}, F_{pas} and nHill were found between cells exchanged with the 3 cTn complexes at both sarcomere lengths. Exchange in IDCM cardiomyocytes with cTn complex containing unphosphorylated cTnI (Wt and 143A) did not affect contractile parameters compared to control (no cTn exchange) IDCM cardiomyocytes (2 IDCM samples; 5 cells) at short and long sarcomere lengths, respectively: F_{max}: 12.2±1.5 and 16.5±2.3 kN/m^2; F_{pas}: 1.5±0.3 and 2.7±0.5 kN/m^2; pCa_{50}: 5.64±0.02 and 5.68±0.02; nHill: 3.2±0.3 and 3.1±0.2.

Table 2. Force measurements in failing cardiomyocytes after exchange with recombinant troponin.

		Wt	143D	143A
F_{max}	1.8 µm	14.5±0.8	13.8±1.3	12.7±1.8
(kN.m^{-2})	2.2 µm	18.0±1.0*	17.2±1.5*	16.8±1.5*
F_{pas}	1.8 µm	2.2±0.1	2.2±0.3	1.9±0.3
(kN.m^{-2})	2.2 µm	3.3±0.3*	3.4±0.4*	3.0±0.3*
pCa_{50}	1.8 µm	5.64±0.02	5.72±0.03$^{\#}$	5.62±0.01
	2.2 µm	5.67±0.02*	5.76±0.02*,$^{\#}$	5.65±0.01*
nHill	1.8 µm	2.9±0.1	3.0±0.3	3.1±0.1
	2.2 µm	2.7±0.1	2.7±0.2	2.8±0.1*

Myofilament force was measured at 1.8 and 2.2 µm sarcomere length and at different Ca^{2+}-concentrations in failing cardiomyocytes (8 myocytes per complex) exchanged with troponin complex mutated at cTnI-Thr143 into alanine (143A; dephosphorylation) or aspartic acid (143D; phosphorylation) and wild-type cTn (Wt). Maximum force (F_{max}) at saturating Ca^{2+}-concentrations, passive force (F_{pas}) and Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) were significantly increased at 2.2 µm compared to 1.8 µm for all complexes. nHill (steepness of the force-pCa curves) was slightly lower at 2.2 µm compared to 1.8 µm in all groups. cTn exchange significantly increased Ca^{2+}-sensitivity in 143D compared to Wt and 143A at both sarcomere lengths. No significant differences between the 3 cTn complexes for F_{max}, F_{pas} and nHill were found at both sarcomere lengths. A two-way ANOVA repeated measures followed by a Bonferroni post-hoc test was used to compare groups exchanged with the different cTn complexes (#P<0.05, significant difference compared to Wt). When the two-way ANOVA revealed a significant effect for sarcomere length (P<0.05), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*P<0.05, 1.8 vs 2.2 µm). Values are means ± SEM.

Overall, these data show that phosphorylation at Thr143 increases myofilament Ca^{2+}-sensitivity, but does not alter LDA of the sarcomeres in failing cardiomyocytes.

Pseudo-phosphorylation of Thr143 and Ser23/24 in donor cardiomyocytes

The absence of an effect of Thr143 phosphorylation in failing cells may be due to a blunted LDA of failing myocardium, as a reduced length-dependent increase in myofilament Ca^{2+}-sensitivity has been reported in failing compared to donor cardiac

tissue (30). In our recent study we found a ΔpCa_{50} of 0.10 ± 0.01 in 6 donor samples (31), which is significantly larger than the value found in the IDCM samples used in this study without cTn exchange (ΔpCa_{50} = 0.04 ± 0.01). Such a blunted length-dependent increase in Ca^{2+}-sensitivity in failing cardiomyocytes may be due to the low baseline cTnI phosphorylation, but could also be related to changes in myofilament proteins other than troponin. Therefore, we studied the effects of Thr143 pseudo-phosphorylation in combination with PKA-mediated effects on LDA in non-failing donor cardiomyocytes. Effects of pseudo-phosphorylation at Thr143 (143D) and at Ser23/24 (23D/24D) were studied without and with treatment with exogenous PKA. The average cTn exchange percentage in donor cardiomyocytes amounted to 82.6 ± 3.1% and did not differ between the 3 complexes (Wt, 143D and 23D/24D). Phosphorylation of other myofilament proteins than cTnI in exchanged donor samples corresponded to: cMyBP-C: 0.57±0.04; desmin: 0.47±0.06; MLC2: 0.28±0.01 and cTnT: 0.24 ± 0.02.

Similar to IDCM, 143D significantly increased Ca^{2+}-sensitivity compared to Wt in 1 donor sample at both sarcomere lengths, while Ca^{2+}-sensitivity was significantly lower compared to Wt in cells exchanged with 23D/24D (Figure 3A-C). No differences were found in ΔpCa_{50} between Wt and 143D, while the length-dependent change in pCa_{50} was higher in cells exchanged with 23D/24D (Figure 3D and Table 3).

The relatively low ΔpCa_{50} in Wt and 143D compared to donor cardiomyocytes without cTn exchange (4 cells; ΔpCa_{50} = 0.10 ± 0.03) and 23D/24D-exchanged cells (Figure 3D) may be explained by removal of phosphorylated endogenous cTnI upon exchange with recombinant unphosphorylated cTn complex. It is known that donor cardiomyocytes are relatively highly phosphorylated at Ser23/24 (10, 39). Phostag analysis showed 52.7% bisphosphorylated, 35.9% monophosphorylated and 11.4% unphosphorylated cTnI at baseline in the donor tissue used in this study (Figure 1A).

Treatment of cTn-exchanged donor cardiomyocytes with exogenous PKA reduced Ca^{2+}-sensitivity in Wt and 143D (Figure 3E) compared to cTn-exchanged cells without PKA treatment (Figure 3C). Moreover, PKA treatment increased ΔpCa_{50} in Wt and 143D (Figure 3F versus Figure 3D). ProQ-Diamond staining illustrated that cTnI and cMyBP-C phosphorylation was 5.5±0.4 and 1.8±0.1 times higher, respectively, in cTn-exchanged donor cells treated with PKA compared to untreated cTn-exchanged donor cells (Figure 3G). This is in line with our previous study (31), where selective PKA-mediated phosphorylation of cMyBP-C and cTnI was found. After PKA treatment, Wt and 143D displayed a similar length-dependent increase in myofilament Ca^{2+}-sensitivity as 23D/24D-exchanged cells (Figure 3F). No effect of PKA-mediated phosphorylation on the length-dependent increase in Ca^{2+}-sensitivity was found in donor cardiomyocytes after exchange with 23D/24D compared to 23D/24D without PKA treatment (Fig 3D, 3F). This most likely demonstrates that PKA-mediated cTnI phosphorylation at Ser23/24 was responsible for the observed enhancement in the length-dependent increase in Ca^{2+}-sensitivity in human donor cardiomyocytes after exchange with Wt and 143D. However, this does not exclude that phosphorylation

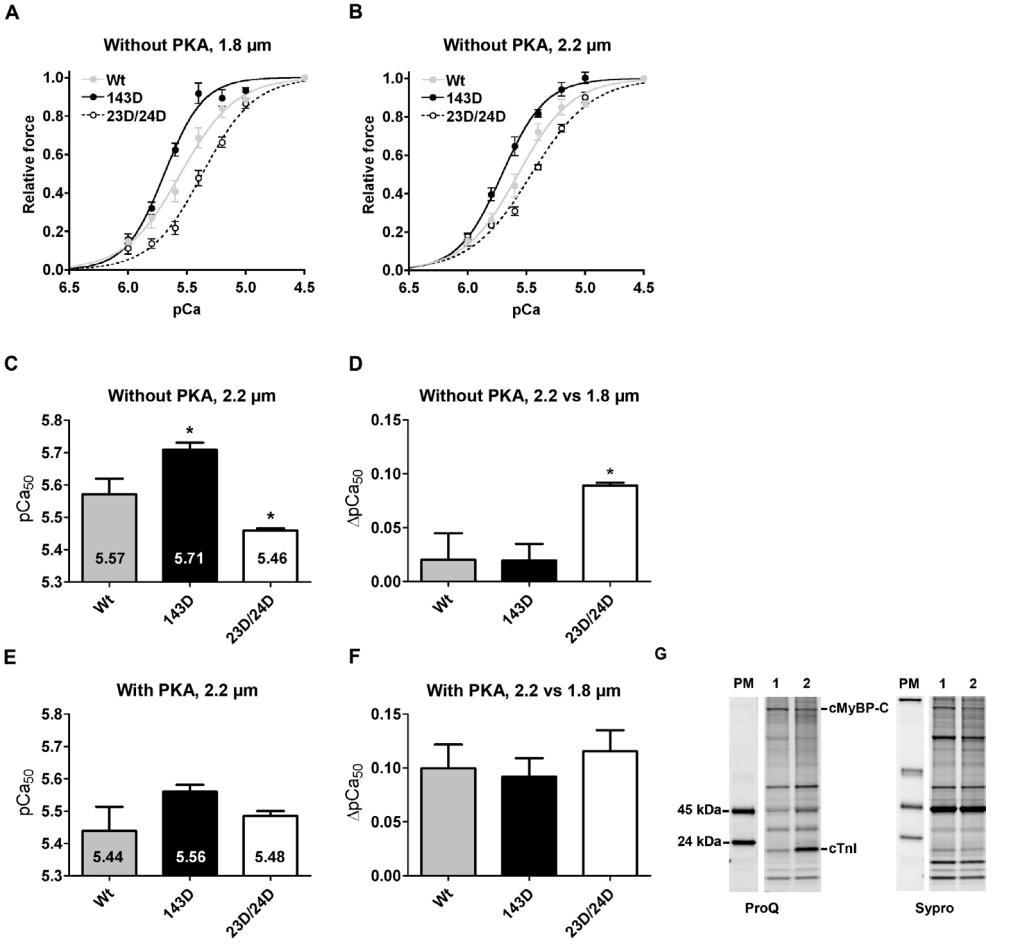

Figure. 3. Phosphorylation of Ser23 and Ser24 regulates the length-dependent increase in Ca^{2+}-sensitivity in donor cardiomyocytes. Myofilament force development was measured at a sarcomere length of 1.8 (A) and 2.2 µm (B) at various [Ca^{2+}] in donor cardiomyocytes in which endogenous troponin complex was partially exchanged (82.6±3.1%) with recombinant myc-tag-labeled troponin complexes (4-5 cardiomyocytes per group). C: Compared to unphosphorylated wild-type cTnI (Wt), Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) was significantly increased after exchange with pseudo-phosphorylated cTnI at Thr143 (143D) and significantly decreased upon pseudo-bisphosphorylation of Ser23/Ser24 (23D/24D) at 2.2 µm. D: The length-dependent increase in Ca^{2+}-sensitivity (ΔpCa_{50}) did not differ between 143D and Wt. However, exchange with 23D/24D significantly increased the length-dependent Ca^{2+}-sensitivity increase compared to Wt. E, F: Troponin-exchanged donor cardiomyocytes were incubated with exogenous PKA. E: PKA treatment decreased Ca^{2+}-sensitivity in cells exchanged with Wt and 143D compared to untreated cardiomyoctes after exchange, but had no effect in 23D/24D cells (Figure 3E versus Figure 3C). F: PKA treatment significantly increased the length-dependent increase in Ca^{2+}-sensitivity for Wt and 143D compared to untreated cells after exchange (Figure 3D versus Figure 3F). No significant differences in ΔpCa_{50} were found after PKA treatment of cells exchanged with the 3 different cTn complexes. *P<0.05, Wt versus 143D and 23D/24D complexes in post-test Bonferroni analyses of one-way ANOVA. PKA treated cardiomyocytes were compared to untreated cardiomyocytes after

exchange with the same complex via a Student's t-test. G: Myofilament proteins of cardiomyocytes after cTn exchanged without (lane 1) and with (lane 2) PKA treatment were separated by 1D-gel electrophoresis. Staining differences between gels were corrected with phosphorylated ovalbumin of the peppermint marker (PM). Phosphorylation signal on ProQ Diamond-stained gels was divided by the SYPRO signal of the same protein to correct for minor differences in loading. Phosphorylation levels of cTnI and cMyBP-C increased after PKA treatment. Phosphorylation of Ser23 and Ser24 regulates the length-dependent increase in Ca^{2+}-sensitivity in donor cardiomyocytes. G: Myofilament proteins of cardiomyocytes after cTn exchanged without (lane 1) and with (lane 2) PKA treatment were separated by 1D-gel electrophoresis. Staining differences between gels were corrected with phosphorylated ovalbumin of the peppermint marker (PM). Phosphorylation signal on ProQ Diamond-stained gels was divided by the SYPRO signal of the same protein to correct for minor differences in loading. Phosphorylation levels of cTnI and cMyBP-C increased after PKA treatment.

of other PKA-mediated phosphorylation targets like titin and cMyBP-C may affect the length-dependent increase in Ca^{2+}-sensitivity, since it is possible that phosphorylation of cMyBP-C and titin exerted opposite effects on the length-dependent change in Ca^{2+}-sensitivity (6, 9).

In line with our experiments in IDCM cardiomyoctes, a sarcomere length increase from 1.8 to 2.2 μm in donor cardiomyocytes without (Table 3) or with (Table 4) PKA treatment increased F_{max} and F_{pas} for all cTn complexes, without changing nHill. Between groups (Wt, 143D and 23D/24D), no significant differences were found for F_{max} and F_{pas} (Table 3 and 4). nHill was significantly higher in 143D compared to Wt at a sarcomere length of 1.8 μm without PKA treatment (Table 3). Overall, our data from failing and non-failing human cardiomyocytes show that cTnI pseudo-phosphorylation at the PKC site Thr143 does not modulate length-dependent myofilament activation.

Both Ser23 and Ser24 need to be phosphorylated to increase length-dependent activation

Our data in donor cardiomyocytes confirm a previous study (16) that indirectly demonstrated that phosphorylation of cTnI-Ser23/24 enhances the increase in Ca^{2+}-sensitivity upon an increase in sarcomere length. However, while monophosphorylation of Ser23 or Ser24 has been reported in the human heart (42, 43), no functional effect of monophosphorylation has been found (39, 44). Therefore, we investigated if monophosphorylation of Ser23 or Ser24 would affect LDA of the myofilaments. For these experiments IDCM cardiomyocytes were used, which showed less than 7% endogenous bisphosphorylation at Ser23/24 (Figure 1A). Due to the low baseline cTnI phosphorylation, the remaining endogenous Ser23/24 bisphosphorylation after cTn exchange does not interfere with functional effects of mono-and bisphosphorylated cTn complexes. IDCM cardiomyocytes (6-9 cells per cTn complex) were exchanged with 4 different pseudo-phosphorylation variants of Ser23/24. The average cTn exchange percentage was 76.6 ± 1.9% and did not differ between the complexes. In accordance with our previous observations in non-failing donor cardiomyocytes (39), only pseudo-bisphosphorylation decreased Ca^{2+}-sensitivity compared to mono-

Table 3. Force measurements in donor cardiomyocytes after exchange with recombinant troponin without PKA treatment.

Without	PKA	Wt	143D	23D/24D
F_{max}	1.8 µm	12.4±0.5	13.3±1.4	11.7±1.1
($kN.m^{-2}$)	2.2 µm	16.2±0.5*	16.7±1.2*	16.3±0.9*
F_{pas}	1.8 µm	2.2±0.3	2.5±0.6	2.9±0.3
($kN.m^{-2}$)	2.2 µm	3.9±0.6*	4.1±0.8*	4.0±0.3*
pCa_{50}	1.8 µm	5.55±0.05	5.69±0.02#	5.37±0.01#
	2.2 µm	5.57±0.05	5.71±0.02#	5.46±0.01*,#
nHill	1.8 µm	1.9±0.1	2.7±0.4#	2.0±0.1
	2.2 µm	1.9±0.1	2.4±0.3	1.7±0.1

One donor sample exchanged with 143D, 23D/24D and Wt troponin complex (4-5 myocytes per complex). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 µm at different Ca^{2+}-concentrations. Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) increased at 2.2 µm compared to 1.8 µm for all complexes, however, the difference was only significant in 23D/24D. Stretching of cardiomyocytes from 1.8 to 2.2 µm increased maximal (F_{max}) and passive force (F_{pas}) in all groups. There was no significant effect of sarcomere length on nHill (steepness of the force-pCa curves). Compared to Wt, 143D significantly increased Ca^{2+}-sensitivity and 23D/24D decreased Ca^{2+}-sensitivity at both sarcomere lengths. No significant differences between the 3 cTn complexes for F_{max} and F_{pas} were found. nHill was significantly higher in 143D compared to Wt only at sarcomere length 1.8 µm. A two-way ANOVA repeated measures followed by a Bonferroni post-hoc test was used to compare groups exchanged with the different cTn complexes (#P<0.05, significant difference compared to Wt). When the two-way ANOVA revealed a significant effect for sarcomere length (P<0.05), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*P<0.05, 1.8 vs 2.2 µm). Values are means ± SEM.

Table 4. Force measurements in donor cardiomyocytes after exchange with recombinant troponin and subsequent PKA treatment.

With	PKA	Wt	143D	23D/24D
F_{max}	1.8 µm	12.8±1.1	11.7±1.1	11.1±1.9
($kN.m^{-2}$)	2.2 µm	14.9±1.6*	14.5±1.6	16.2±2.2*
F_{pas}	1.8 µm	2.0±0.2	2.0±0.2	2.6±0.1
($kN.m^{-2}$)	2.2 µm	2.9±0.2*	3.4±0.5*	3.6±0.3*
pCa_{50}	1.8 µm	5.34±0.06	5.47±0.01#	5.37±0.01
	2.2 µm	5.44±0.08*	5.56±0.02*	5.48±0.01*
nHill	1.8 µm	2.1±0.1	2.3±0.2	1.7±0.1
	2.2 µm	2.1±0.1	2.4±0.2	1.7±0.2

One donor sample exchanged with 143D, 23D/24D and Wt troponin complex with PKA treatment (4-5 myocytes per complex). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 µm at different Ca^{2+}-concentrations. Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa50) significantly increased at 2.2 µm compared to 1.8 µm for all complexes. Stretching of cardiomyocytes from 1.8 to 2.2 µm increased maximal (Fmax) and passive force (Fpas) in all groups, without affecting nHill (steepness of the force-pCa curves). Compared to Wt, 143D increased Ca^{2+}-sensitivity, reaching significance at sarcomere length 1.8 µm (#). pCa50 of Wt did not significantly differ from 23D/24D after PKA treatment. No significant differences between the 3 cTn complexes for Fmax, Fpas and nHill were found. A two-way ANOVA repeated measures followed by a Bonferroni post-hoc test was used to compare groups exchanged with the different cTn complexes (#P<0.05, significant difference compared to Wt). When the two-way ANOVA revealed a significant effect for sarcomere length (P<0.05), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*P<0.05, 1.8 vs 2.2 µm). Values are means ± SEM.

and dephosphorylated Ser23/24 in IDCM cardiomyocytes (Figure 4A). Compared to pseudo-dephosphorylated Ser23/24 (23A/24A), pseudo-monophosphorylation at Ser23 (23D/24A) or Ser24 (23A/24D) did not affect LDA (Figure 4B and Table 5). Upon exchange with pseudo-bisphosphorylated Ser23/24 (23D/24D), the length-dependent increase in Ca^{2+}-sensitivity was significantly increased compared to 23A/24A (Figure 4B). The enhanced length-dependent increase in Ca^{2+}-sensitivity in 23D/24D was significantly higher compared to control IDCM cardiomyocytes without exchange. The sarcomere length increase from 1.8 to 2.2 μm increased F_{max} and F_{pas} for all cTn complexes, without changing nHill (Table 5).

Length-dependent myofilament activation and titin-based passive stiffness

Titin-based passive stiffness has been shown to exert a profound effect on length-dependent activation. A high expression of the stiff N2B isoform in cardiac tissue has been associated with a higher passive force and larger length-dependent increase in myofilament Ca^{2+}-sensitivity compared to tissue with a high expression of compliant N2BA isoform (9). Thus, changes in titin isoform composition, as have been reported in human heart failure (21, 23), may alter length-dependent activation. In our exchange experiments, passive tension did not differ between the different experimental groups (Tables 2-5). Moreover, no differences were observed in F_{pas} and titin isoform composition (Figure 5) between the failing and donor samples used in the present study.

DISCUSSION

This study demonstrates that cTnI pseudo-phosphorylation at the PKC-site Thr143 increases myofilament Ca^{2+}-sensitivity without affecting the length-dependent increase in maximal force or Ca^{2+}-sensitivity in human failing and non-failing donor cardiomyocytes. The length-dependent increase in Ca^{2+}-sensitivity is enhanced by (pseudo-)phosphorylation of Ser23/24 only upon phosphorylation of both sites.

Phosphorylation of Thr143 increases Ca^{2+}-sensitivity without affecting length-dependent activation

Tachampa et al. (33) showed that substitution of a single amino acid at position 143 of cTnI (from Thr to Pro) greatly diminished the length-dependent increase in myofilament Ca^{2+}-sensitivity. Since Thr143 is a well-known PKC-mediated phosphorylation site that has been implicated in heart failure (25, 32, 43), we investigated if phosphorylation of Thr143 may modify LDA of the myofilaments in human cardiomyocytes. We show that pseudo-phosphorylation of Thr143 does not affect the length-dependent increase in maximal force and Ca^{2+}-sensitivity irrespective of the level of cTnI phosphorylation at PKA-sites.

The finding that Thr143 phosphorylation does not affect LDA is not in conflict with the previous finding that a substitution of Thr143 by a proline, as present in ssTnI and

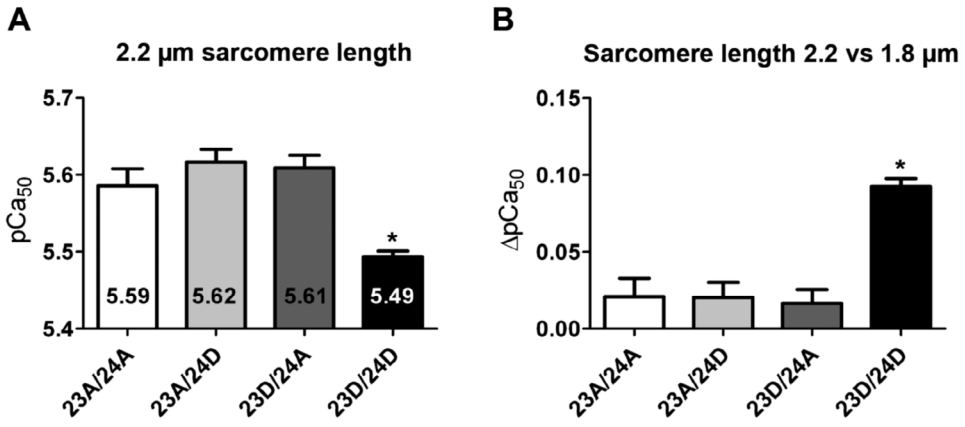

Figure 4. Phosphorylation of both Ser23 and Ser24 is required to increase length-dependent activation in failing cardiomyocytes. Myofilament force development was measured at sarcomere lengths of 1.8 and 2.2 µm at various [Ca^{2+}] in failing cardiomyocytes in which endogenous troponin complex was partially exchanged (76.6±1.9%) with recombinant myc-tag-labeled troponin complexes (6-9 cells per group). A: Compared to pseudo-unphosphorylated Ser23/24 (23A/24A), Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) decreased upon pseudo-bisphosphorylation of Ser23 and Ser24 (23D/24D), while no effect was seen with mono-phosphorylated complexes (23A/24D and 23D/24A). B: The length-dependent increase in Ca^{2+}-sensitivity was not changed compared to pseudo-unphosphorylated Ser23/24 (23A/24A) upon pseudo-monophosphorylation of Ser23 or Ser24 (23D/24A, 23A/24D). Pseudo-bisphosphorylation of Ser23 and Ser24 enhanced the length-dependent Ca^{2+}-sensitivity increase compared to 23A/24A. *, $P<0.05$, 23A/24A versus 23A/24D, 23D/24A and 23D/24D complexes in post-test Bonferroni analyses of one-way ANOVA.

fast skeletal TnI, severely blunts LDA in cardiac muscle. A proline is a neutral amino acid with very specific properties (reviewed by Williamson (40)). Replacement of a Thr by a Pro, alters the amino acid charge differently compared to threonine phosphorylation. Likewise, mutations in the inhibitory region of cTnI, which are associated with inherited forms of cardiomyopathies, may disrupt LDA. We recently reported blunted LDA in myocardium from patients with hypertrophic cardiomyopathy harboring a mutation (R145W) in cTnI (31). Overall, these results indicate that mutation-induced changes in amino acids in the inhibitory region of cTnI and phosphorylation-mediated changes of Thr143 exert different effects on sarcomere function. While the threonine at position 143 is key for LDA in cardiac muscle, phosphorylation at this site does not modulate LDA.

Pseudo-phosphorylation of Thr143 using aspartic acid at amino acid 143 increased Ca^{2+}-sensitivity both in IDCM and donor cardiomyocytes. In contrast, exchange in mouse fibers with pseudo-phosphorylated Thr143 mimicked by a glutamate did not change (5) or non-significantly decreased Ca^{2+}-sensitivity (32) compared to Wt exchange. Our data are in line with a study of Wang et al. where incorporation of phosphate instead of pseudo-phosphorylation was used (38). By using genetically

Table 5. Force measurements in failing cardiomyocytes after exchange with recombinant troponin mutated at Ser23 and Ser24.

		23A/24A	23A/24D	23D/24A	23D/24D
F_{max}	1.8 µm	12.4±0.6	11.9±0.5	13.2±0.5	11.5±0.7
($kN.m^{-2}$)	2.2 µm	15.4±0.7*	17.3±1.0*	17.7±0.8*	14.5±0.7*
F_{pas}	1.8 µm	2.2±0.4	1.7±0.2	1.5±0.3	1.9±0.2
($kN.m^{-2}$)	2.2 µm	2.8±0.6*	2.2±0.3*	2.1±0.3*	2.2±0.2*
pCa_{50}	1.8 µm	5.57±0.02	5.60±0.01	5.59±0.02	5.40±0.01#
	2.2 µm	5.59±0.02	5.62±0.02	5.61±0.02	5.49±0.01*,#
nHill	1.8 µm	2.4±0.2	2.1±0.1	2.2±0.1	2.0±0.1
	2.2 µm	2.2±0.3	1.9±0.1	2.2±0.1	2.1±0.2

Failing cardiomyocytes exchanged with 23A/24A, 23A/24D, 23D/24A and 23D/24D (6-9 cells per group). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 µm at different Ca^{2+}-concentrations. Ca^{2+}-sensitivity derived from the midpoint of the force–pCa relationship (pCa_{50}) increased at 2.2 µm compared to 1.8 µm for all complexes, however, the increase was only significant in 23D/24D. Stretching of cardiomyocytes from 1.8 to 2.2 µm significantly increased maximal (F_{max}) and passive force (F_{pas}) in all groups. There was no significant effect of sarcomere length on nHill (steepness of the force-pCa curves). Ca^{2+}-sensitivity was significantly lower in 23D/24D compared to 23A/24A at both sarcomere lengths (#, $p<0.05$). No significant differences between the 4 cTn complexes for F_{max}, F_{pas} and nHill were found at both sarcomere lengths. A two-way ANOVA repeated measures followed by a Bonferroni post-hoc test was used to compare groups exchanged with the different cTn complexes (#$P<0.05$, significant difference compared to 23A/24A). When the two-way ANOVA revealed a significant effect for sarcomere length ($P<0.05$), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*$P<0.05$, 1.8 vs 2.2 µm). Values are means ± SEM.

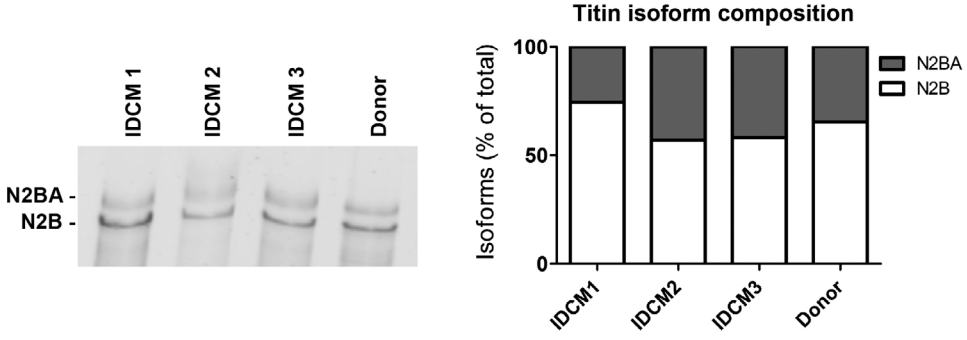

Figure 5. Titin isoform composition in the IDCM and donor tissue. Titin isoform composition was analyzed in the IDCM and donor samples. A SYPRO-stained gel shows the compliant N2BA and stiff N2B titin isoform. On average, titin composition in the IDCM samples did not differ from the donor sample.

modified mice harboring alanine mutations at the different phosphorylation sites of cTnI, they demonstrated that PKC-βII preferentially phosphorylates Thr143 and thereby increased Ca^{2+}-sensitivity. This suggests that an aspartic acid pseudo-

phosphorylation at Thr143 more closely resembles PKC-mediated phosphorylation of Thr143 compared to a glutamate pseudo-phosphorylation.

The absence of an effect of Thr143 pseudo-phosphorylation on LDA is in line with the preserved LDA at the myofilament level in a mouse model of heart failure induced by over-expression of PKCε (20), which has been demonstrated to phosphorylate Thr143 (15). Thr143 phosphorylation did not increase the relatively small length-dependent increase in Ca^{2+}-sensitivity when Ser23/24 are largely unphosphorylated in failing cells. One possibility to explain this may be the elevated Ca^{2+}-sensitivity, limiting further Ca^{2+}-sensitivity enhancement upon lengthening. However, pseudo-phosphorylation of Thr143 in combination with phosphorylation of Ser23/24 also did not change the enhanced length-dependent increase in Ca^{2+}-sensitivity. In donor cardiomyocytes, Wt without PKA showed a pCa_{50} of 5.57 ± 0.05 and a relatively low length-dependent increase in Ca^{2+}-sensitivity (Figure 3C,D), while 143D after PKA displayed the same pCa_{50} (5.56 ± 0.02) with an enhanced length-dependent Ca^{2+}-sensitivity increase (Figure 3E,F). These observations support the conclusion that an enhanced length-dependent increase in Ca^{2+}-sensitivity is not directly related to a lower myofilament Ca^{2+}-sensitivity induced by phosphorylation of Ser23/24. This is in line with our previous study where a low Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm was found in a patient with a cTnI mutation (R145W) after treatment with exogenous PKA together with a blunted length-dependent increase in Ca^{2+}-sensitivity (31). Titin isoform composition did not differ between the samples used for exchange experiments and therefore most likely did not contribute to the changes observed in length-dependent myofilament activation in our exchange experiments. In summary, our data show that PKC-mediated phosphorylation at Thr143 sensitizes the myofilament to Ca^{2+} without affecting length-dependence of the sarcomeres independent of the initial value of Ca^{2+}-sensitivity which is set by the phosphorylation status of other troponin sites and sarcomeric proteins.

Phosphorylation of both Ser23 and Ser24 increases length-dependent activation

Exchange with wild-type troponin complex did not alter the length-dependent increase in Ca^{2+}-sensitivity in IDCM cardiomyocytes compared to (unexchanged) control cardiomyocytes, whereas it significantly reduced the length-dependent increase in Ca^{2+}-sensitivity in donor cardiomyocytes compared to control cardiomyocytes. An important difference in cTn between donor and IDCM cardiomyocytes is that donor cardiomyocytes are highly bisphosphorylated at Ser23/24 (>50%), in contrast to end-stage IDCM (<10%) (10, 39). LDA has been reported to be regulated by PKA-mediated phosphorylation of cTnI at these sites, although a reduction (14) and enhancement (16) of the length-dependent change in Ca^{2+}-sensitivity has been reported upon PKA incubation. In addition, contrasting results have been reported when pseudo-phosphorylated cTnI at Ser23/24 was exchanged, showing a blunted (28) or enhanced (24) change in length-dependent Ca^{2+}-sensitivity compared to Wt exchange. The reason for these differences is unclear, however, one possible

explanation for the blunted length-dependent increase in Ca^{2+}-sensitivity that was found in previous studies may be that a different protocols was used (14, 28). In the present study we first performed all force measurements at a low sarcomere length of 1.8 μm and thereafter performed measurements at 2.2 μm. We did not stretch cardiomyocytes to a sarcomere length of 2.3 μm to prevent damage to the sarcomeres. However, Rao et al. (28) and Kajiwara et al. (14) first measured at a sarcomere length of 2.3 μm and thereafter at a shorter sarcomere length of 2.0 μm.

In this study, we demonstrate using PKA-mediated phosphorylation and the exchange of pseudo-phosphorylated Ser23/24, that phosphorylation of Ser23/24 enhances the length-dependent increase in Ca^{2+}-sensitivity in human cardiomyocytes because: 1) Exchange with Wt complex removed endogenous phosphorylation at Ser23/24 in donor cardiomyocytes and blunted the length-dependent increase in Ca^{2+}-sensitivity compared to baseline measurements in donor cells. 2) PKA treatment after Wt exchange restored the length-dependent increase in Ca^{2+}-sensitivity in donor cardiomyocytes. 3) Exchange with pseudo-phosphorylated Ser23/24 preserved the length-dependent increase in Ca^{2+}-sensitivity in donor cardiomyocytes and enhanced the length-dependent increase in Ca^{2+}-sensitivity in IDCM cardiomyocytes to levels observed in donor. In support, it has been demonstrated that PKA-mediated phosphorylation shifts a shallow length-tension relationship to a steeper length-tension relationship in rat cardiomyocytes (12). In accordance, exchange with pseudo-phosphorylated Ser23/24 in rat cardiomyocytes was associated with a steepened length-tension relationship providing evidence that phosphorylation of cTnI-Ser23/24 is essential to enhance length-dependent activation (11). In addition, we demonstrated using mono-and bisphosphorylated cTn complexes that phosphorylation at both Ser23 and Ser24 is required to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity.

Implication of cTnI phosphorylation at Thr143 and Ser23/24 and in health and disease

In the present study, we provide evidence that phosphorylation of the PKC site Thr143 does not affect LDA, while bisphosphorylation of the PKA-sites Ser23/24 enhances the length-dependent increase in Ca^{2+}-sensitivity in human cardiomyocytes (Figure 6). In the healthy heart, sympathetic activation during stress and exercise increases heart rate and stroke volume to meet the demands of the body. This is accomplished via activation of β_1-adrenergic receptors, which leads to activation of PKA. PKA phosphorylation of Ca^{2+} handling proteins and myofilament proteins enhances cardiomyocyte contraction and relaxation (for reviews: (3, 35)). The enhanced length-dependent increase in Ca^{2+}-sensitivity induced upon PKA phosphorylation of cTnI-Ser23/24 will increase the range of myofilament Ca^{2+}-sensitivities at which the heart can operate within a heart cycle (between short and long sarcomere lengths) as is illustrated in figure 6 (Wt versus Wt+PKA, 23D/24D, 23D/24D+PKA which resembles activation of the heart upon β-adrenergic receptor stimulation).

In end-stage heart failure the Frank-Starling relationship has been reported to be depressed (13, 29). LDA is believed to be the cellular basis of the Frank-Starling mechanism (26, 34). Schwinger et al. previously reported a reduced length-dependent increase in Ca^{2+}-sensitivity in skinned fibers from terminally failing human myocardium (30). In our study, replacement of endogenous cTn with pseudo-bisphosphorylated cTnI at Ser23/24 enhanced the length-dependent increase in myofilament Ca^{2+}-sensitivity in failing cardiomyocytes (Figure 4B). This suggests that the blunted length-dependent increase in myofilament Ca^{2+}-sensitivity which has been reported previously (30) in end-stage heart failure may be at least partly caused by reduced bisphosphorylation at Ser23/24 of cTnI relative to explanted donor tissue, which has been reported in several studies (4, 18, 36, 42).

Apart from reduced PKA-mediated cTnI phosphorylation, increased PKC-mediated phosphorylation of Thr143 may modify LDA of cardiac muscle in heart failure since this amino acid has been demonstrated to play a key role in the length-dependent increase in Ca^{2+}-sensitivity (33). Pseudo-phosphorylation of the PKC site Thr143 did not affect LDA properties of human heart muscle, but did increase Ca^{2+}-sensitivity as illustrated in Figure 6 (Wt versus 143D, Wt+PKA versus 143D+PKA). Based on our results, PKC-mediated phosphorylation of Thr143, which has been implicated in cardiac disease (25, 32, 43), does not restore the blunted length-dependent Ca^{2+}-sensitivity increase in the failing heart, although it does increase Ca^{2+}-sensitivity. The high Ca^{2+}-sensitivity may aid to maintain cardiac output of the failing heart, however, it may also contribute to diastolic dysfunction.

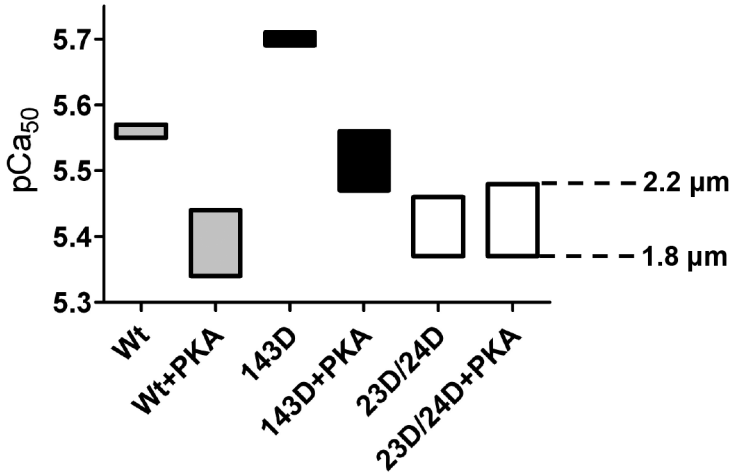

Figure 6. Titin isoform composition in the IDCM and donor tissue. Titin isoform composition was analyzed in the IDCM and donor samples. A SYPRO-stained gel shows the compliant N2BA and stiff N2B titin isoform. On average, titin composition in the IDCM samples did not differ from the donor sample.

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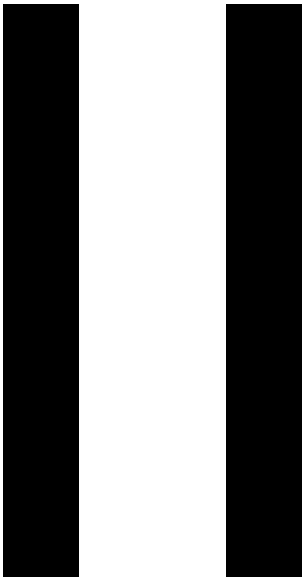
No conflicts of interest, financial or otherwise, are declared by the author(s).

Author contributions

Author contributions: P.J.M.W., V.S., D.B.F., Y.L. performed experiments; P.J.M.W., V.S., G.J.M.S., J.V.D.V. analyzed data; P.J.M.W., V.S., D.B.F., Y.L., C.G.D.R., A.M.M., G.J.M.S., J.V.D.V. approved final version of manuscript; P.J.M.W., A.M.M., G.J.M.S., J.V.D.V. conception and design of research; P.J.M.W., V.S., D.B.F., Y.L., C.G.D.R., A.M.M., G.J.M.S., J.V.D.V. interpreted results of experiments; P.J.M.W. prepared figures; P.J.M.W., V.S., D.B.F., Y.L., C.G.D.R., A.M.M., G.J.M.S., J.V.D.V. drafted manuscript; P.J.M.W., G.J.M.S., and J.V.D.V. edited and revised manuscript.



MYOCARDIAL ADP ACCUMULATION AND CONTRIBUTION OF CROSS-BRIDGE FORMATIONS TO DIASTOLIC DYSFUNCTION

5

SYNERGISTIC ROLE OF ADP AND Ca^{2+} IN DIASTOLIC MYOCARDIAL STIFFNESS

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"Perhaps they will consider, however, these few generalizations, which I know are true: Being right isn't enough. What you say, however right, must be said in a currently acceptable language, must not violate too brutally current taste, and must somehow signal your membership in a respectable professional club. If you want to succeed you must play all of the game, not just the innings in laboratory or library."

Paul Gross

KEY POINTS

Diastolic dysfunction in heart failure patients is evident from stiffening of the passive properties of the ventricular wall.

Increased actomyosin interactions may significantly limit diastolic capacity, however direct evidence is absent.

From experiments at the cellular and whole organ level, in humans and rats, we show that actomyosin-related force development contributes significantly to high diastolic stiffness in environments where high ADP and increased diastolic [Ca^{2+}] are present, such as the failing myocardium.

Our basal study provides a mechanical mechanism which may partly underlie diastolic dysfunction.

ABSTRACT

Heart failure (HF) with diastolic dysfunction has been attributed to increased myocardial stiffness that limits proper filling of the ventricle. Altered cross-bridge interaction may significantly contribute to high diastolic stiffness, but this has not been shown thus far. Cross-bridge interactions are dependent on cytosolic [Ca^{2+}] and the regeneration of ATP from ADP. Depletion of myocardial energy reserve is a hallmark of HF leading to ADP accumulation and disturbed Ca^{2+}-handling. Here, we investigated if ADP elevation in concert with increased diastolic [Ca^{2+}] promotes diastolic cross-bridge formation and force generation and thereby increases diastolic stiffness. ADP dose-dependently increased force production in the absence of Ca^{2+} in membrane-permeabilized cardiomyocytes from human hearts. Moreover, physiological levels of ADP increased actomyosin force generation in the presence of Ca^{2+} both in human and rat membrane-permeabilized cardiomyocytes. Diastolic stress measured at physiological lattice spacing and 37°C in the presence of pathological levels of ADP and diastolic [Ca^{2+}] revealed a 76±1% contribution of cross-bridge interaction to total diastolic stress in rat membrane-permeabilized cardiomyocytes. Inhibition of creatine kinase (CK), which increases cytosolic ADP, in enzyme-isolated intact rat cardiomyocytes impaired diastolic re-lengthening associated with diastolic Ca^{2+}-overload. In isolated Langendorff-perfused rat hearts, CK-inhibition increased ventricular stiffness only in the presence of diastolic [Ca^{2+}]. We propose that elevations of intracellular ADP in specific types of cardiac disease, including those where myocardial energy reserve is limited, contribute to diastolic dysfunction by recruiting cross-bridges even at low Ca^{2+} and thereby increase myocardial stiffness.

ABBREVIATIONS

HF, Heart failure; CK, Creatine kinase; PCr, Phosphocreatine; LVEDP, Left ventricle end-diastolic pressure; IDCM, idiopathic dilated cardiomyopathy; F_{pas}, Passive tension; ADP-F_{total}, ADP-stimulated total tension; ADP-F_{act}, ADP-active tension; Ca^{2+}-F_{total}, Ca^{2+}-stimulated total tension; Ca^{2+}-F_{act}, Ca^{2+}-active tension; EC_{50}, ADP- or Ca^{2+}-sensitivity (at which half of F_{act} is reached); ktr, Ca^{2+}-ADP dependent rate of force redevelopment; PV, Pressure-volume; DNFB, 2,4-dinitro-1-fluorobenzene; SERCA, Sarcoplasmic reticulum Ca^{2+}-ATPase

INTRODUCTION

Heart failure (HF) is a clinical syndrome defined as the inability of the heart to sufficiently supply blood to organs and tissues.[1] While contractile dysfunction is present in about half of the HF patient population almost all patients show impaired diastolic function.[1,2] Pathophysiological mechanisms which have been related with diastolic dysfunction involve alterations of the passive properties of the ventricular wall, including high titin-based myofilament passive stiffness and collagen deposition, and slow rates of myocardial relaxation due to increased myofilament Ca^{2+}-sensitivity and impaired Ca^{2+}-reuptake.[3-5]

Depletion of myocardial energy reserve represents a major cause of dysfunction of the failing human heart.[6] In the healthy heart, creatine kinase (CK) catalyzes the transfer of phosphate from phosphocreatine (PCr) to ADP, thereby regenerating ATP, while preventing accumulation of cytosolic ADP.[7] ATP is essential for cross-bridge cycling. As little as 0.1 mM ATP is sufficient for cross-bridge detachment and sarcomere relaxation.[8] The relatively high myocardial *in vivo* ATP levels in both healthy (8-11 mM) and various pathological conditions (7-10 mM)[9-11] indicate that reductions in ATP levels are unlikely to directly impair relaxation of the heart muscle. Notably, HF patients with diastolic dysfunction[12,13] and animal models of HF[10,11,14,15] show that the reduced PCr/ATP ratio (a parameter of myocardial energy reserve measured *in vivo*) is mostly explained by a severe reduction in PCr (up to 50%) rather than changes of ATP (≤25%). Reducing PCr or experimental inhibition of CK activity is causally linked to the development of HF, as it elevates left ventricle end-diastolic pressure (LVEDP), reduces contractile reserve and increases mortality in rats.[14-16] Activity of CK isoforms is decreased by 50% in animal models of HF.[17-19] The consequence of low PCr and/or a reduced CK activity is that cytosolic levels of ADP will increase. Indeed, increases in [ADP] of >50% have been reported in several animal models of HF.[9-11] Selectively increasing ADP levels without altering cytosolic ATP levels has been shown to increase LVEDP and limit myocardial relaxation in rats.[9,16] These studies support the idea that limited myocardial energy reserve via elevations of ADP may represent a likely cause of diastolic dysfunction. However the exact pathophysiological mechanism has not been elucidated.

Intriguingly, ADP can stimulate tension generation, in the absence of Ca^{2+}, in membrane-permeabilized rabbit skeletal muscle fibers[20] and bovine cardiac muscle[21,22]. These findings indicate that ADP accumulation, as a consequence of disturbed myocardial energy reserve, may lead to incomplete relaxation during diastole caused by residual actomyosin interactions. However, in HF, the accumulation of ADP occurs concomitantly with disturbed Ca^{2+}-handling.[23-25] The effects of elevated intracellular ADP and high Ca^{2+} levels on human myofilament function that exist during the diastolic phase are currently not known. We hypothesize that in failing hearts enhanced cross-bridge formation and force generation, as a result of increased levels of ADP and diastolic [Ca^{2+}] increase diastolic stiffness.

METHODS

Ethical Approval

Human samples were obtained after written informed consent and with approval of St Vincent's Hospital (#H03/118) and from the Human Research Ethics Committee of the University of Sydney (#159401). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997). All animal experiments were performed with approval of the Animal Care and Use Committee of the VU University Medical Center (Amsterdam) and UK Home Office and local ethical review.

Myocardial human samples

Human left ventricular (LV) tissue was obtained during heart transplantion surgey from end-stage failing patients with idiopathic dilated cardiomyopathy (IDCM). Tissue was immediately frozen and stored in liquid nitrogen.

Isometric force measurements in membrane-permeabilized single human cardiomyocytes

Small human cardiac tissue samples (N=3) were thawed in relaxing solution and cardiomyocytes were mechanically isolated by tissue disruption as described previously.[26] Cardiomyocytes were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a piezoelectric motor. Isometric force measurements were subsequently performed on single cardiomyocytes at a sarcomere length of 2.2 µm. Absolute force values were normalized to cardiomyocyte cross-sectional area and expressed as developed tension (in kN/m^2). Passive tension (F_{pas}) was determined by allowing the cardiomyocyte to shorten by 30% of its length in relaxing solution. ADP-stimulated total tension was determined by activating the cardiomyocyte at 10 mM ADP (ADP-F_{total}, Figure 1A). Similarly, Ca^{2+}-stimulated total tension was determined by activating the cardiomyocyte at 32 µM Ca^{2+} (Ca^{2+}-F_{total}; Figure 1A). Active tension (Ca^{2+}-F_{act} or ADP-F_{act}) was calculated as $F_{act}=F_{total}-F_{pas}$. In addition, the rate of force redevelopment (ktr) was determined using a slack-restretch test as follows: after steady state force was reached, cardiomyocytes were allowed to shorten to 70% of their original length within 1 ms and then re-stretched to the original length after 30 ms. A single exponential was fitted to determine the rate constant of force redevelopment: ktr was determined at maximal and submaximal [Ca^{2+}] in the presence of ADP to assess cross-bridge cycling kinetics.

Experimental solutions

The composition of all solutions was calculated based on a computer program similar to that described previously.[27] The pH of all solutions was adjusted to 7.1 at 15°C with KOH and ionic strength was adjusted to 180 mM with KCl. The relaxing solution contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and 14.5 mM PCr

(P7936, Sigma). Several activating solutions were prepared: 1) Ca^{2+}-activating solution consisted of 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and 32 µM free Ca^{2+}. Ca^{2+}-activating solutions with lower free [Ca^{2+}] were obtained by mixing of the Ca^{2+}-activating and relaxing solutions and assuming an apparent stability constant of the Ca^{2+} -EGTA complex of $10^{6.35}$; 2) ADP-activating solutions (in the absence of free Ca^{2+}) contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and MgADP in increasing concentrations from 1 to 10 mM; 3) Ca^{2+}- activating solutions in the presence of ADP consisted of 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES, MgADP concentrations from 0.02 to 6 mM and free Ca^{2+} from 1 to 32 µM. PCr was omitted from the activating solutions to preserve the MgADP/MgATP ratio. All force measurements were performed at 15°C. [ATP] was maintained constant at 1 mM to avoid rigor cross-bridge formation (reported to occur when [ATP] falls below 0.1 mM[8]) and consecutively increased the [ADP] from 1 to 10 mM. We used a maximum [ADP] of 10 mM, since ADP-stimulated tension was maximal at 10 mM ADP with 1 mM ATP in bovine cardiac muscle[21,22].

Data analysis of isometric force measurements

Ca^{2+}- and/or ADP-force relations were determined by a non-linear fit procedure using a modified Hill equation using KaleidaGraph version 3.6 (Synergy Software, Reading, PA) as follows accordingly:

$$P\,(Ca^{2+}) / P_0 = [Ca^{2+}]^{nH} / (K^{nH} + [Ca^{2+}]^{nH})$$

$$P\,(ADP) / P_0 = [ADP]^{nH} / (K^{nH} + [ADP]^{nH})$$

$$P\,(Ca^{2+}\text{-}ADP) / P_0 = [Ca^{2+}\text{-}ADP]^{nH} / (K^{nH} + [Ca^{2+}\text{-}ADP]^{nH})$$

where P is steady-state force. P_0 denotes the steady isometric force at either saturating [Ca^{2+}], high [ADP] or both saturating [Ca^{2+}] and high [ADP], nH describes the steepness of the relationship, and K represents the [Ca^{2+}] and/or [ADP] at which force is half-maximal (0.5 x P_0). Myofilament Ca^{2+}- and/or ADP-sensitivity is denoted as EC_{50}.

Isolation of intact rat ventricular cardiomyocytes

Intact rat cardiomyocytes were isolated using collagenase digestion of hearts as described previously.[28] Briefly, adult wild-type male Wistar rats (N=7) weighing ~300 grams were euthanized with isoflurane and hearts were quickly removed and rinsed in cold isolation-Tyrode solution (composition: 130 mM NaCl, 5.4 mM KCl, 3 mM NaPyruvate, 25 mM HEPES, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO4, 22 mM glucose) containing 0.2 mM EGTA (Tyrode-EGTA) and pH 7.4. The heart was then cannulated via the aorta to the Langendorff apparatus and perfused for 2 min with Tyrode-EGTA at 37°C. Thereafter, the heart was perfused with enzyme-Tyrode solution consisting of

Tyrode solution, 1.2 mg/ml collagenase (Type II, 265 U/mg; Worthington Biochemical, NJ, USA) and 50 μM $CaCl_2$ for a period of 7 min. The right ventricle and atria were removed, and the LV was cut into small pieces and triturated with a plastic Pasteur pipette for 3 min in stopping buffer solution 1 (SB-1; composition: Tyrode solution, 0.6 mg/ml collagenase, 100 μM $CaCl_2$ and 10 mg/ml bovine serum albumin (BSA)). The cell suspension was filtered through a 300 μm nylon mesh filter into a 50 ml Falcon tube and centrifuged for 1 min at 27x g (20-23°C). The pellet containing cardiomyocytes was resuspended in SB solution 2 (SB-2; composition: Tyrode solution, 250 μM $CaCl_2$ and 10 mg/ml BSA) and incubated for 10 min in a water bath at 37°C allowing cells to settle. The supernatant was discarded and cardiomyocytes were resuspended in SB solution 3 (SB-3; containing Tyrode solution, 500 μM $CaCl_2$ and 10 mg/ml BSA), incubated for 10 min at 37°C and subsequently resuspended in experimental Tyrode (used for measurements of intact cardiomyocytes), consisting of 1.2 mM $CaCl_2$, 137 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO4, 0.57 mM $MgCl_2$, 5 mM HEPES and 5.6 mM glucose. Cardiomyocytes were used within the first 6h of isolation.

Isometric force measurements in membrane-permeabilized single rat cardiomyocytes

Single cardiomyocytes isolated by collagenase digestion from adult wild-type male Wistar rats (N=4) were used. Cardiomyocytes were chemically permeabilized with 0.5% (v/v) Triton-X100 as indicated above for human and isometric force measurements were performed at sarcomere lengths of 2.2 μm. Experimental temperature and solution conditions were similar as previously described for human samples. Ca^{2+}-force relations without and with ADP, and the rate of cross-bridge cycling was determined as previously described for human samples.

Diastolic stress measurements in membrane-permeabilized rat cardiomyocytes

Single cardiomyocytes isolated by collagenase digestion from adult wild-type male Wistar rats (N=5) were used. Cardiomyocytes were chemically permeabilized with 0.5% (v/v) Triton-X100 as indicated above and isometric force measurements were performed in single cardiomyocytes stretched from 1.8 to 2.4 μm sarcomere lengths. Experiments were performed at 15°C to replicate conditions currently employed to study diastolic stiffness/stress and at a higher, more physiological temperature of 37°C. Since membrane-permeabilization causes myofilament lattice expansion, experiments were also performed with the osmotic agent dextran T500 (4%, w/v) that compresses the myofilament lattice to physiological levels.[29] Additionally, 30 μM of the cross-bridge inhibitor blebbistatin was used to assess the contribution of cross-bridge formation to passive stress. Force generation was normalized to cardiomyocyte cross-sectional area and expressed as diastolic stress (in kN/m^2). The composition and characterization of all 4 conditions is displayed in Table 1. To mimic conditions currently in use to study diastolic stiffness/stress, experiments were performed at 15°C in the 1) absence and 2) presence of blebbistatin. To approach *in vivo* disease conditions

Table 1. Solution's composition of the 4 conditions tested.

Condition	[Ca^{2+}] (μM)	Dextran	Blebbistatin (μM)	PCr (mM)	MgATP (mM)	MgADP (μM)
Unphysiological						
15°C	<0.001	-	-	14.5	10	-
15°C+Blebbistatin	<0.001	-	30	14.5	10	-
Physiological						
37°C	0.15	4%	-	-	10	100
37°C+Blebbistatin	0.15	4%	30	-	10	100

PCr, Phosphocreatine; pH was adjusted to 7.1 at 15°C and at 37°C with KOH and ionic strength was adjusted to 180 mM with KCl; 2 mM free Mg^{2+}, 10 mM MgATP, 7 mM EGTA and 100 mM BES were present in all solutions; Experiments performed at 15°C were used to mimic conditions currently employed to study diastolic stiffness, while experiments at 37°C better recapitulate the *in vivo* diseased heart.

measurements were performed at 37°C and with Dextran T500 in the 3) absence and 4) presence of blebbistatin. Because blebbistatin is photosensitive, experiments were performed in the dark. To ensure whether total absolute tension values were absent of protocol artefacts due to substantial differences in cross-sectional area that could artificially increase or decrease diastolic stress, all cross-sectional measurements were done in solutions without dextran. Cross-sectional area did not statistically differ between conditions 1 to 4 (342.8±18, 370.1±39, 354±22 and 350±36 kN/m^2/μm, respectively).

Cardiomyocyte shortening and Ca^{2+}-transient of intact rat cardiomyocytes

Unloaded shortening and Ca^{2+}-transients of intact rat cardiomyocytes (n=15 to 20) were monitored following field stimulation (1Hz, 4 ms, 7V), and sarcomere shortening as well as relaxation kinetics were visualized using a video-based sarcomere length (SL) detection system (IonOptix corporation). Wild-type rat cardiomyocytes were incubated in Tyrode solution containing 1 μM Fura-2-AM for 15 min and rinsed for a period of 10 min in Tyrode. Cardiomyocytes were placed into a temperature-controlled (37°C) chamber with platinum electrodes to electrically stimulate cells. Single cardiomyocytes were randomly selected (without spontaneous contraction) and continuously perfused with Tyrode buffer. After 3-5 min field stimulation sarcomere shortening as well as relaxation kinetics and Ca^{2+}-transient were monitored. Cardiomyocytes loaded with Fura-2-AM were excited at 340 and 380 nm with emission monitored at 510 nm. The F340/F380 ratio was used as a measure of cytosolic [Ca^{2+}]. From the monitored Ca^{2+}-transient, Ca^{2+}-release and -reuptake velocity, the time to reach peak systolic Ca^{2+} and the time constant of Ca^{2+} decline (tau) were determined.

Experimental protocol for unloaded rat cardiomyocyte measurements

To test the hypothesis that ADP accumulation impairs myofilament function in cardiomyocytes with intact membrane, a protocol was established using a low dose of an irreversible inhibitor of CK, iodoacetamide. Specifically, 5 mM fresh iodoacetamide (I6125, Sigma) was dissolved in Tyrode buffer and perfused (at 1 ml/min) for 3 min, delivering a total dose of 15 μmoles/3 ml. This drug has well-characterized effects on the ATP-regeneration system, such that it significantly increases [ADP] without altering ATP, PCr, inorganic phosphate and pH levels in isolated perfused hearts.[9,16] ADP is estimated to be lower than 73 μM at this dose range.[16]

Two protocols were used to test the effects of CK-inhibition (Fig. 5). Cardiomyocytes were paced at 1Hz for 3-5 min with continuous perfusion with Tyrode solution (at 1 ml/min and 36±0.1°C) to establish a steady state. Thereafter, a number of cells was either perfused for 3 min with a total dose of 15 μmoles/3 ml of iodoacetamide (Fig. 5A) or continuously perfused with Tyrode solution (Fig. 5C). Following 3 min treatment with iodoacetamide, perfusion with Tyrode solution was restored. Perfusion was limited to 3 min/ml with 5 mM of iodoacetamide, because the Ca^{2+}-Fura signal deteriorated by longer exposure and/or greater concentration of drug. Control experiments to check for Ca^{2+}-fluorescence demonstrated that iodoacetamide does not quench Ca^{2+} (data not shown). Measurements were averaged and analyzed in three phases as detailed in the legend of Figure 5. The control protocol was the same as the treatment protocol except that cells were continuously perfused with Tyrode solution.

Isolated perfused Langendorff hearts: Diastolic pressure-volume relationships

Adult wild-type male Wistar rats were euthanized by stunning and cervical dislocation and hearts quickly removed and rinsed in cold Krebs-Henseleit (KH) solution. Diastolic pressure-volume relationships were measured in hearts connected to a Langendorff apparatus and perfused with Ca^{2+}-free KH solution gassed with 95% O_2-5% CO_2 at 37°C and pH 7.4. An inflatable balloon (filled with pre-boiled, degassed Milli-Q water) was placed in the left ventricle and the volume adjusted with a syringe pump dispensing at 400 μL/min. Balloon volume was inferred from dispensing rate and pressure was recorded continuously via a side port connected to a pressure transducer. Balloon volume was increased in cycles to elicit a series of pressures from 10-30 mmHg allowing the balloon to conform to the LV cavity surface; the final cycle to 30 mmHg was repeated and all diastolic pressure-volume data were obtained from it.[30]

The experimental procedures were as follows: diastolic pressure-volume relationship of Langendorff-perfused rat hearts were recorded after 60 min perfusion with Ca^{2+}-free KH with (N=5 hearts) or without (N=6 hearts) 20 μM of the cell-permeant Ca^{2+}-chelator BAPTA-AM (Cambridge Bioscience, UK) dissolved in DMSO (final concentration <0.1%). Perfusion solution was then switched to a Ca^{2+}-free KH containing 20 μM 2,4-dinitro-1-fluorobenzene (DNFB; 42085, Sigma) dissolved in ethanol (final concentration <0.1%), for 30 min then PV measurements were repeated. DNFB is an irreversible inhibitor of CK which decreases the ATP/

ADP ratio.[31] Because iodoacetamide inhibits enzymes of the glycolytic reaction (even though well-oxygenated hearts have normal glycolysis after iodoacetamide-treatment[16]), whereas DNFB does not[32], we wanted to ensure complete preservation of the glycolytic flux following long chemical inhibition of a multicellular system such as the whole heart. DNFB has been shown to produce similar effects in isolated cardiomyocytes as iodoacetamide.[33]

Creatine Kinase activity

CK activity levels were assessed using a commercial colorimetric assay kit (ab155901, Abcam) that detects kinase activity as low as 1 mU/ml (nmol/min/ml). Activity was assessed in freshly isolated cell suspensions from rat cardiomyocytes (N=3) as follows: from each single animal, two suspensions of isolated cells were treated (i.e. one treated with buffer (control) and another with 5 mM iodoacetamide) for 5 minutes, centrifuged for 30 seconds at 27x g (37°C) and supernatant discarded. The pellet containing cardiomyocytes was immediately frozen in liquid nitrogen and freeze-dried overnight. Assay was performed as specified by manufacturer supplied information. Similarly, CK activity was evaluated in fresh rat cells from 3 animals without and in the presence of 20 µM of DNFB.

Statistical analysis

Data analysis and statistics were performed using Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA) and SAS/STAT® 9.2 (Statistical Analysis System Software, Institute Inc., Cary, NC, USA).

Data are presented as mean ± SEM of all single cardiomyocytes per patient/animal or group. All data was tested for normality using the Shapiro-Wilk Test. Normality was assumed when $p>0.05$ and the variances were equal. Paired- and/or unpaired-sample Student's t-Test was conducted when one variable was tested between 2 groups of data. Single classification analysis of variance (ANOVA) was performed when one variable was tested in more than 2 groups. When a significant difference was found in repeated measures 1-way ANOVA, Bonferroni *post hoc* testing was used to determine the location of significant differences. When more than one variable was tested in more than 2 groups a 2-way ANOVA was conducted followed by a Bonferroni *post hoc* test if significant differences were found. A linear mixed model was performed to gain insights into the individual trajectories of repeated measurements of each data group (e.g. intercepts and slopes) compared with controls. This model takes into account the repeated measurements within subjects. Each performed test is specified in Tables or Figure legends. The level of significance was set at $p<0.05$.

RESULTS

ADP stimulates myofilament contraction in the absence of Ca^{2+}

To assess whether ADP alone can induce contraction in human cardiac muscle, force produced by single membrane-permeabilized cardiomyocytes from human failing hearts was measured at increasing [ADP]. ADP caused force production in the absence of Ca^{2+} with maximal tension (ADP-F_{act}) achieved with 10 mM ADP and tension reaching 80±3% of maximal Ca^{2+}-developed tension (Fig. 1A). Comparable to Ca^{2+}, force-ADP relation curves show the common sigmoidal pattern, indicating cooperative activation (Fig. 1B). ADP-induced tension at 1 mM ADP amounted to 14±1% and nearly saturated at 8 mM ADP (94±3%). Half-maximal ADP-F_{act} (EC_{50}) was 3.80±0.15 mM ADP (Fig. 1B).

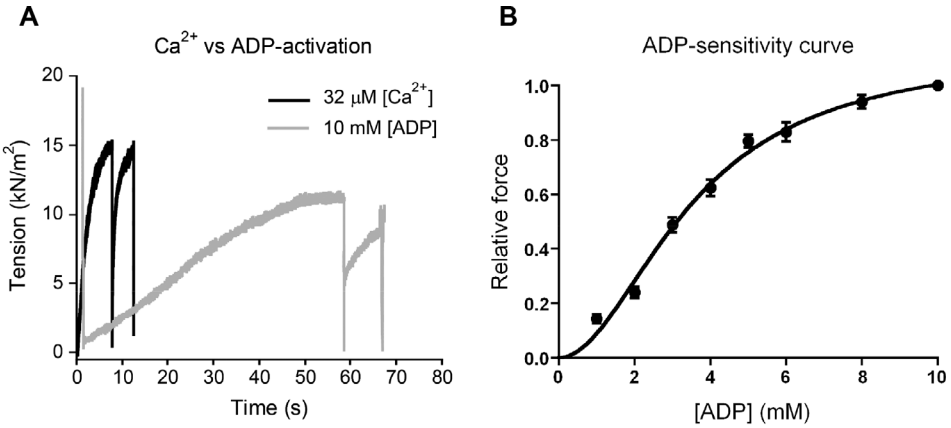

Figure 1. ADP causes force production in human left ventricular membrane-permeabilized cardiomyocytes. A, Representative tension recordings of a single cardiomyocyte isolated from a end-stage failing human heart at 2.2 µm sarcomere length during maximal Ca^{2+} (32 µM) and ADP-activation (10 mM) at 15°C and 1 mM ATP. B, Normalized force-ADP relationship of human cardiomyocytes. 9 to 11 cardiomyocytes from 3 failing human hearts were used.

ADP increases myofilament Ca^{2+}-sensitivity

Myocardial ADP levels in animal models are reported in the range of 10-50 µM in health and of 40-140 µM in disease[9-11], indicative that *in vivo* levels of ADP are incapable of myocardial activation since *in vitro* mM levels of ADP are required for force generation (Fig. 1B). Cardiomyocytes *in vivo* are activated by the cytosolic Ca^{2+} transient that varies from ~0.15 µM during diastole to a peak of ~1.6 µM in systole.[34]

We therefore investigated if elevation of ADP in concert with physiological levels of Ca^{2+} increases force generation in human membrane-permeabilized cardiomyocytes (Fig. 2A). Physiological levels of ADP were chosen in the range of 20 and 100 µM. ADP

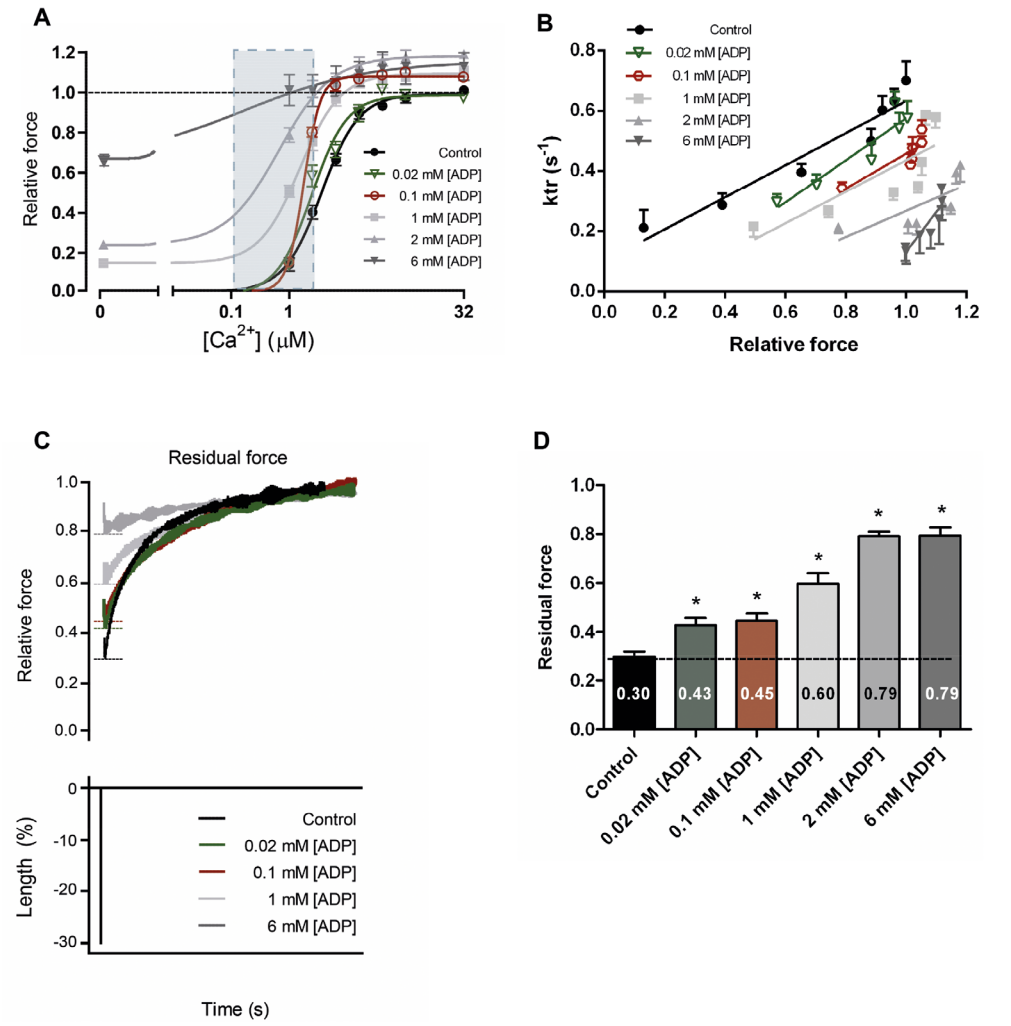

Figure 2. Ca^{2+}-sensitivity and cross-bridge stiffness increases in the presence of ADP in human left ventricular membrane-permeabilized cardiomyocytes. A, Ca^{2+}-force relations were constructed without and with [ADP] at 15°C and 1 mM ATP. Light blue panel depicts the free [Ca^{2+}] range in *in vivo* cardiomyocytes (i.e. [~0.15-1.6 μM]). Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. All data show significant differences compared with 'control (no [ADP])'. B, Normalization of ktr as a function of Ca^{2+}-activated isometric force. The ktr-force relationship shifted to the right and downwards with [ADP] (R^2=0.93, R^2=0.88, R^2=0.76, R^2=0.65, R^2=0.55 and R^2=0.81, from 0-6 mM ADP respectively). A linear mixed model assuming force as a continuous variable indicates significant differences for the interactions of 2 (p=0.020) and 6 mM (p<0.0001) ADP compared with 'control (no [ADP])'. C, Residual force recording tracings. Top: Residual force recordings after a slack-restretch test at high Ca^{2+} (32 μM) without or with increasing [ADP]. Recording of 2 mM ADP is omitted from the graph due to overlap with 6 mM ADP recording. Bottom: Length changes of piezoelectric motor by 30% of cardiomyocyte's original length is depicted. Residual force was calculated from the initial force recovery reached after the length change (dashed lines) and normalized to each total steady-state force reached before length change. Measurements are normalized to each corresponding total force. D, Significant increases in residual force enhancement

- at high Ca^{2+} without or with various [ADP] are observed after a slack-restretch test, indicative for increased sarcomere stiffness. Values are normalized to each corresponding total relative force reached before the slack-restretch procedure. Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. $^{*}p<0.05$ versus 'control (no [ADP])'. 9 to 11 cardiomyocytes from 3 end-stage human failing hearts were used for each ADP concentration.

shifted the Ca^{2+}-force relation substantially to the left, indicative of increased Ca^{2+}-sensitization of myofilaments (Fig. 2A). Indeed, as little as 20 μM ADP significantly increased Ca^{2+}-F_{act} generation, evident from the elevated Ca^{2+}-sensitivity compared with the Ca^{2+}-force relation without ADP (EC_{50}=1.69±0.10 and 1.95±0.09 μM Ca^{2+}, respectively). Our data suggests that conditions that promote elevation of myocardial ADP result in cross-bridge recruitment even at low Ca^{2+}, supporting delayed muscle relaxation.

ADP slows cross-bridge detachment in the presence of Ca^{2+}

The rate of myofilament contraction and relaxation is regulated by the rate of attachment and detachment of force producing cross-bridges, i.e. cross-bridge cycling. Therefore, the rate of force redevelopment (ktr, a measure of the rate of cross-bridge cycling) in the presence of Ca^{2+} and ADP was examined, and values were plotted against the corresponding isometric force (Fig. 2B). When Ca^{2+}-activated isometric force rises with increasing [ADP] the ktr relationships shifted substantially to the right and downwards with significant differences found for relationships with supra-physiological ADP levels present (≥2 mM ADP). This suggests that force increases at the expense of slowing cross-bridge cycling (Fig. 2B), likely resulting from a population of strongly-bound cross-bridges with slow detachment kinetics. This is consistent with significant increases in sarcomere stiffness (i.e. high residual force recovery) at [ADP] ≥20 μM, indicative for reduced actomyosin detachment and enhanced strain/force per each individual cross-bridge (Figs. 2C and 2D).

To test whether the latter changes in myofilament Ca^{2+}-sensitivity, cross-bridge cycling and residual force recovery (Fig. 2) are species-independent, similar experiments were performed in rat membrane-permeabilized cardiomyocytes in the presence of 100 μM ADP. Rat membrane-permeabilized cardiomyocytes present similar dependence to ADP as humans, such as increased myofilament Ca^{2+}-sensitization, slower rates of cross-bridge cycling and high residual force recovery (Fig. 3).

Together these data indicate that in membrane-permeabilized cardiomyocytes the combined presence of Ca^{2+} and physiological ADP levels increases cross-bridge stiffness, even at low Ca^{2+} levels, which could contribute to high sarcomere stiffness and reduce LV compliance during the diastolic phase.

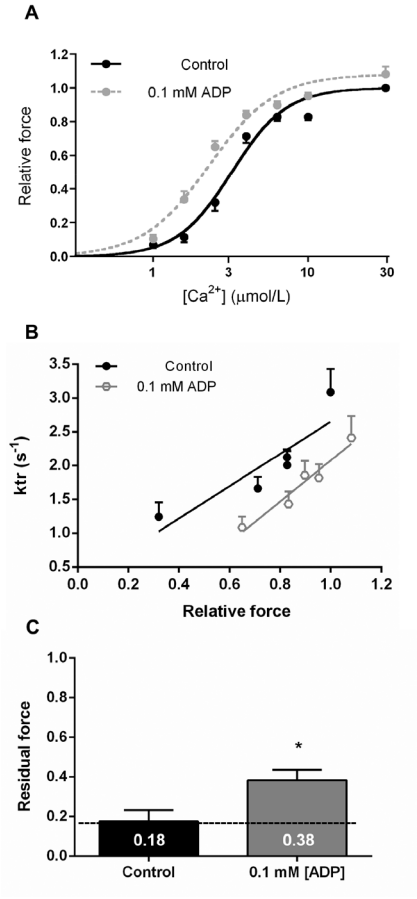

Figure 3. Ca^{2+}-sensitivity and cross-bridge stiffness increases in the presence of ADP in left ventricular rat membrane-permeabilized cardiomyocytes. A, Ca^{2+}-force relations were constructed without and with 0.1 mM ADP at sarcomere lengths of 2.2 µm and at 15°C and 1 mM ATP. Data was compared using paired t-Test. Ca^{2+}-sensitivity of force significantly increases in the presence of 0.1 mM ADP compared with 'control (no [ADP])'. B, Normalization of ktr as a function of Ca^{2+}-activated isometric force. The ktr-force relationship shifted to the right and downwards with 0.1 mM ADP (R^2=0.79 and R^2=0.95, 'control (no [ADP])' and 0.1 mM ADP, respectively). C, Significant increases in residual force enhancement at high Ca^{2+} with 0.1 mM ADP are observed after a slack-restretch test compared with 'control (no [ADP])', indicative for increased sarcomere stiffness. Values are normalized to each corresponding total relative force reached before the slack-restretch procedure. Data was compared using paired t-Test. *p<0.05 versus 'control (no [ADP])'. 12 cardiomyocytes from 4 adult wild-type male Wistar rat hearts were used.

Re-evaluation of the influence of cross-bridges on *in vitro* diastolic stress with *in vivo* conditions

In the last decade, numerous *in vitro* studies assessed the diastolic properties of human HF myocardium and no evidence was found for the possible contribution of cross-bridges to the high diastolic stiffness observed in disease.[3,5] Nevertheless,

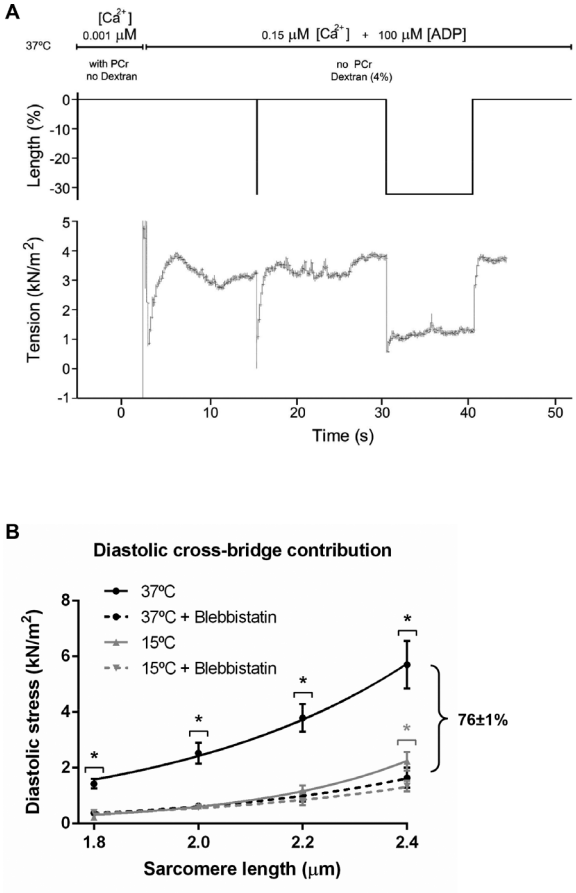

Figure 4. Re-evaluation of the influence of cross-bridges on *in vitro* diastolic stress with *in vivo* conditions. A, Representative force recording example of a left ventricular single rat cardiomyocyte moved from a non-activating solution at 37°C with 14.5 mM PCr and <0.001 µM Ca^{2+} to an activating solution at 37°C in the presence of diastolic Ca^{2+} (0.15 µM), dextran (4%), 100 µM ADP and 10 mM ATP. Upper tracing: solutions used in the protocol are depicted. Middle: Length changes of piezoelectric motor is depicted. For measurement of passive force, a slack-restretch procedure of 30% of cardiomyocyte's original length is performed. Bottom: Force recording after a slack-restretch test is depicted. Diastolic tension value was taken from the 30% slack procedure over a period of 10 seconds (period from 30 to 40 seconds). B, Tension-sarcomere length relations are depicted from left ventricular rat membrane-permeabilized cardiomyocytes. Rat cardiomyocytes were stretched from 1.8 to 2.4 µm and diastolic stress was measured in the following conditions: 37°C (Dextran (4%), diastolic Ca^{2+} (0.15 µM) and 100 µM ADP) in the absence (dark exponential curve) and presence of Blebbistatin (37°C + Blebbistatin; dashed dark exponential curve); and 15°C (with 14.5 mM PCr and <0.001 µM Ca^{2+}) in the absence (gray exponential curve) and presence of Blebbistatin (15°C + Blebbistatin; dashed gray exponential curve). All solutions contained 10 mM ATP. Comparison of 37°C with 37°C+Blebbistatin shows that the cross-bridge contribution accounts for 76±1% of total diastolic stress averaged over all sarcomere lengths. Data was compared using unpaired t-Test within independent groups measured at similar temperature for each individual sarcomere length. *p<0.05 versus 'in the presence of Blebbistatin'. 12 to 16 cardiomyocytes from 5 adult wild-type male Wistar rat hearts were used.

the majority of the studies thus far have been based on membrane-permeabilized myocytes, where the relatively low experimental temperature (15°C) in concert with sarcolemma digestion that causes lattice expansion and removes important differences in intracellular milieu (such as increased ADP levels), may largely underestimate cross-bridge interactions. To overcome some of these concerns we adjusted the protocol previously reported by King et al.[28], where diastolic stress was measured in rat membrane-permeabilized cardiomyocytes at 37°C in the presence of the osmotic agent dextran to compress myofilament lattice restoring it to physiological conditions. By measuring cardiomyocyte diastolic stress, in the presence of diastolic [Ca^{2+}] and [ADP] known to occur in disease with and without the cross-bridge inhibitor blebbistatin (30 μM), we were able to assess the contribution of cross-bridge formation to diastolic myocardial stress/stiffness. Please note that for the initial experiments in membrane-permeabilized cardiomyocytes from human (Fig. 2) and rat (Fig. 3) the temperature was set at 15°C. This relatively low temperature was used to avoid preparation deterioration and maintain the stability of the muscle preparation during several long-lasting series of activation. This was shown not to be problematic for the current experiments at 37°C as a result of lower levels of activation in concert with a relatively brief protocol.

Four protocols were performed in which rat membrane-permeabilized cardiomyocytes were stretched from 1.8 to 2.4 μm sarcomere length. Conditions used are presented in Table 1. A representative recording is presented in Figure 4A showing diastolic force generation measured at 37°C. Note that force is generated at 37°C, which is not seen in the other three conditions. Comparison of 15°C and 15°C+Blebbistatin confirmed the previous observations that cross-bridge contribution to diastolic stiffness is nearly absent at non-physiological conditions, with a statistical difference found at a sarcomere length of 2.4 μm (Fig. 4B). However, comparison of 37°C with 37°C+Blebbistatin shows that the cross-bridge contribution is significantly higher, accounting for 76±1% of total diastolic stress averaged over all sarcomere lengths (Fig. 4B). Together these data support that in intracellular environments where high ADP and increased diastolic [Ca^{2+}] are present, such as in the failing myocardium, the actomyosin contribution to high diastolic stiffness may be substantially higher than previously considered and potentially represent a pathophysiological mechanism of HF with impaired diastolic function.

CK-inhibited rat cardiomyocytes show impaired relaxation and augmented contractility

To investigate the impact of inactivated CK activity and the consequent increase in cytosolic ADP levels, as to replicate the cytosolic environment of HF myocardium, intact rat cardiomyocytes were perfused with the irreversible CK antagonist iodoacetamide (15 μmoles/3 ml) during pacing at 1Hz and 37°C. CK activity was inhibited by 38±7%. Contraction and Ca^{2+}-transient were simultaneously recorded in control and iodoacetamide-treated intact rat cardiomyocytes (Fig. 5). No significant alterations were observed between controls and iodoacetamide group at baseline (phase I)

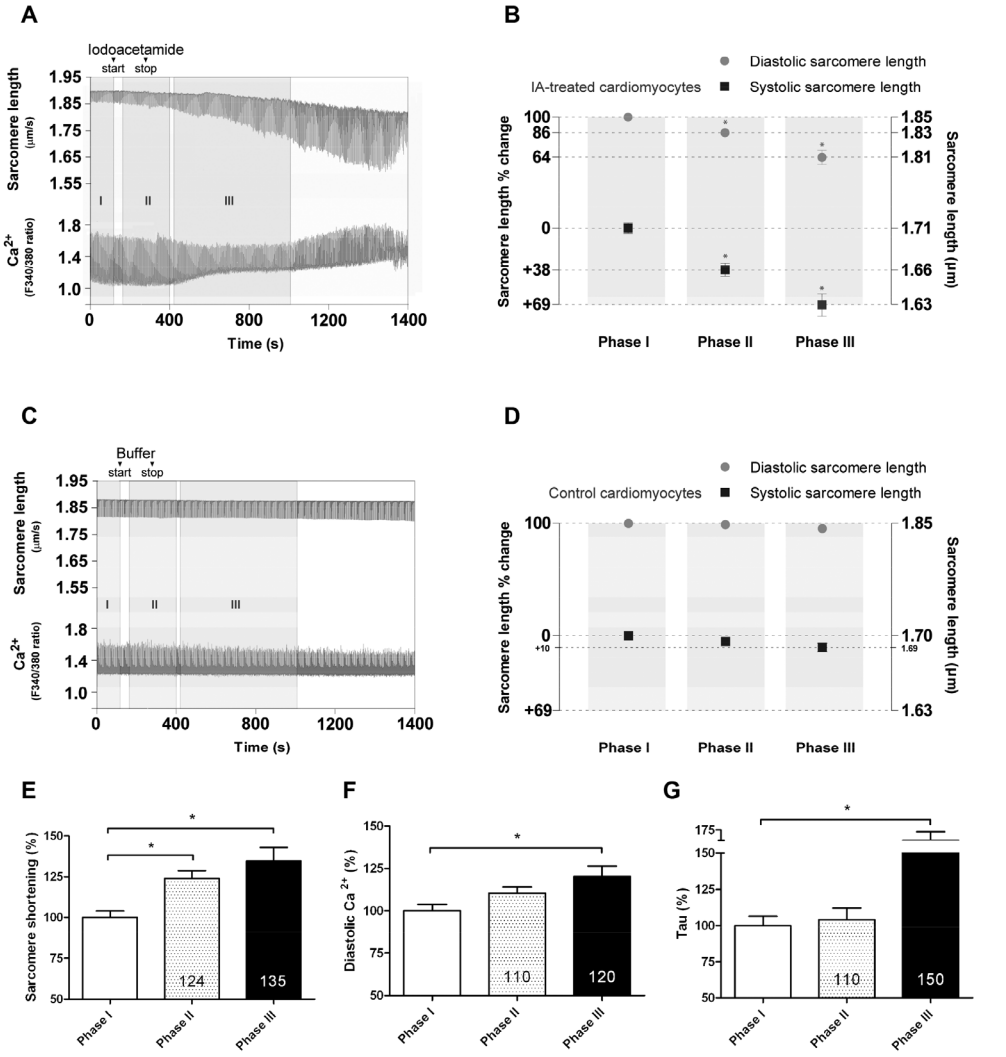

Figure 5. Creatine kinase-inhibited cells show decreased diastolic length but augmented contractility in intact left ventricular rat cardiomyocytes. Example of sarcomere length and Ca^{2+}-transient recordings of left ventricular single unloaded intact rat cardiomyocytes with iodoacetamide treatment at 1Hz pacing. A, Iodoacetamide treatment. Phase (I). Baseline. Measurements just prior to iodoacetamide administration (cardiomyocytes paced for 3-5 minutes). Iodoacetamide (start-stop). Perfusion (at 1 ml/min) was performed for 3 min with 5 mM iodoacetamide resulting in a total dose of 15 µmoles/3 ml. Phase (II). Initial perfusion with iodoacetamide. Cardiomyocytes were allowed 1 min to adjust to iodoacetamide perfusion and measurements were recorded for 4 min. Phase (III). Post-iodoacetamide perfusion. Measurements were carried out ranging from 3-10 min depending on cardiomyocytes survival. Phase III was typified by diastolic [Ca^{2+}] increases as well as systolic and diastolic sarcomere length alterations. Note: example of cell death in the last 100 seconds of recording. B, Alterations of diastolic and systolic sarcomere length from phase I (baseline), throughout phase II (initial perfusion with iodoacetamide) to phase III (after iodoacetamide perfusion), in iodoacetamide (IA)-treated cells. Relative sarcomere length changes compared with phase I of each corresponding group. *versus Phase I. C, Control recording protocol (no treatment) tracing. Similar to (A) except,

instead of iodoacetamide treatment, cardiomyocytes were perfused with buffer solution. Note: during both protocols cardiomyocytes were continuously perfused with buffer solution (at 1 ml/min) with the exception being the 3 minute period of iodoacetamide perfusion in (A). D, Alterations of diastolic and systolic sarcomere length from phase I (baseline), throughout phase II (similar perfusion time as phase II in (B)) to phase III (similar perfusion time as phase III in (B)), in control cells. Relative sarcomere length changes compared with phase I of each corresponding group. E, % increase change of sarcomere shortening. F, % increase of diastolic Ca^{2+}-level. G, % delay of the Ca^{2+}-relaxation time constant, tau, indicative of Ca^{2+}-reuptake. Figures 5E, 5F and 5G are all % changes relative to phase I in IA-treated cells. Corresponding data are presented in Table 2. $^{*}p<0.05$ versus Phase I. Data was compared using 1-way ANOVA followed by Bonferroni *post hoc* test. 7 heart samples from adult wild-type male Wistar rats were used; 17 to 18 (Ctrl-group) and 17 to 20 (IA-group) cardiomyocytes were analysed.

(Table 2 and Figs. 5A-5G). Control cardiomyocytes remained stable throughout the entire protocol for all parameters analyzed, i.e. no significant alterations were observed, (compared from phase I to phase III) with the exception for decreases of systolic and amplitude Ca^{2+}-signal, likely resulting from deterioration of the fluorescence signal over time (Table 2).

From phase I (baseline) to phase II (initial perfusion with iodoacetamide), diastolic and systolic sarcomere length were significantly reduced by 14±2% and 38±6%, respectively (Fig. 5B and Table 2), and sarcomere shortening increased by 24±5% (Fig. 5E and Table 2). In addition, cardiomyocyte shortening time was significantly increased without a significant change in shortening velocity (Table 2). The contractile changes were not mediated by changes in Ca^{2+}-transients, since there were no statistical alterations in diastolic Ca^{2+} (Fig. 5F) and the Ca^{2+}-relaxation time constant (tau) (Fig. 5G) from phase I to II.

Between phases II and III (after iodoacetamide perfusion) diastolic and systolic sarcomere lengths decreased significantly compared with baseline and phase II (Fig. 5B and Table 2). This was accompanied by 35±8% enhancement of sarcomere shortening compared with baseline (Fig. 5E). These latter contractile changes are associated with disturbed Ca^{2+}-handling, which includes significant increases of diastolic Ca^{2+} (20±6%, Fig. 5F) with prolongation of Ca^{2+}-reuptake by 50±20% (tau) (Fig. 5G) compared with baseline (Table 2).

Together these data indicate that inhibiting CK, which elevates cytosolic ADP, in intact cardiomyocytes leads to impaired diastolic relaxation (i.e. limited diastolic re-lengthening). Initially, these alterations are independent of changes in the Ca^{2+} transient, but prolonged CK-inhibition was associated with diastolic Ca^{2+}-overload and slowing of Ca^{2+}-reuptake.

CK-inactivation decreases ventricular compliance in the presence of diastolic Ca^{2+}

Tian et al.[9,16] have previously shown that myocardial ADP elevation increases LVEDP and impairs myocardial relaxation in isolated rat hearts. However, the authors argued against a role of Ca^{2+} and solely attributed the latter effects to high [ADP] [16]. To clarify whether ADP is the sole responsible factor for the reported increase in LVEDP,

ventricular compliance was measured in isolated Langendorff-perfused rat hearts, in which CK was inactivated, in the absence and presence of the intracellular Ca^{2+}-chelator BAPTA-AM. Diastolic pressure-volume (PV) relationships were performed in hearts arrested in Ca^{2+}-free buffer following inhibition of CK with 2,4-dinitro-1-fluorobenzene (DNFB) and with BAPTA-AM (Fig. 6A). DNFB (20 μM) inhibited CK activity reaction by 42±8%, i.e. about equal to iodoacetamide effect. Diastolic PV slope increased in hearts perfused with DNFB in Ca^{2+}-free solution (Fig. 6B), indicative of reduced LV compliance. In other hearts, addition of BAPTA-AM (20μM) to Ca^{2+}-free solution (-Ca^{2+}+BATPA) did not change diastolic PV slope compared to -Ca^{2+} solution (Fig. 6B), but prevented the increase in diastolic PV slope following DNFB (Fig. 6B). To understand whether Ca^{2+} alone could account for the increased PV slope, 200 μM of caffeine was added to induce Ca^{2+}-release (Fig. 6B). The PV slope only showed a modest increase compared to CK-inhibition (Fig. 6B). Together, these results indicate that the combined presence of CK-inactivation and diastolic [Ca^{2+}] are needed for the observed decline in ventricular compliance in whole heart preparations.

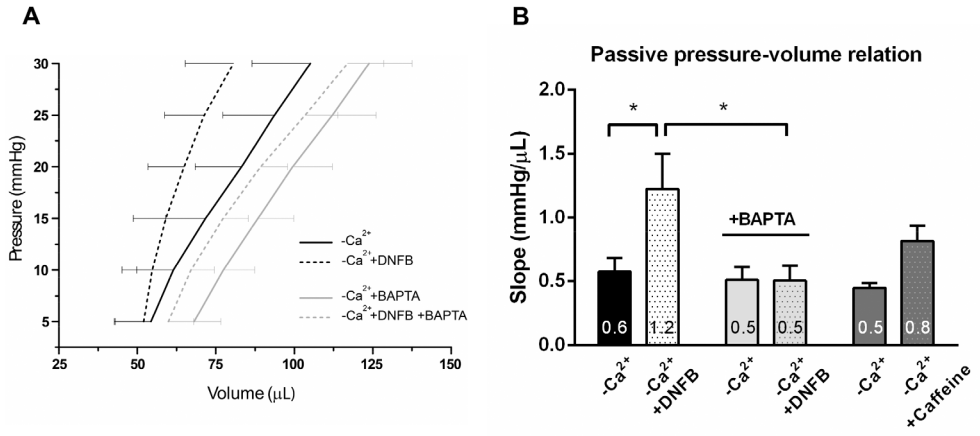

Figure 6. Left ventricle (LV) compliance declines following creatine kinase (CK)-inactivation in isolated rat hearts. A, Mean diastolic pressure-volume (PV) relationship in Langendorff-perfused hearts arrested in Ca^{2+}-free buffer without and with inhibition of CK. Hearts (N=6) were perfused with nominally Ca^{2+}-free solution for 60 min then the diastolic PV relationship was measured (-Ca^{2+}). 2,4-dinitro-1-fluorobenzene (DNFB; 20 μM) was then perfused for a further 30 min and measurements repeated (-Ca^{2+}+DNFB). In a separate group of hearts (N=5), the intracellular Ca^{2+}-chelator BAPTA-AM (20μM) was added to -Ca^{2+} solution and perfused for 60 min (-Ca^{2+}+BAPTA), followed by perfusion for 30 min with DNFB (-Ca^{2+}+DNFB+BAPTA). B, Slope of the diastolic PV relationship. DNFB reduced left ventricle compliance in -Ca^{2+} solution, shown by a steeper diastolic PV relationship. BAPTA did not change diastolic PV slope compared to -Ca^{2+}. However, BAPTA prevented the increase in diastolic PV slope following DNFB perfusion. In a separate group of hearts (N=2), addition of low concentration of caffeine (200 μM) in Ca^{2+}-free solution appears to be sufficient to cause diastolic Ca^{2+}-leak, indicative from the increase in diastolic PV slope. Although to a lesser extent than -Ca^{2+}+DNFB. Data was compared using 2-way repeated measures ANOVA followed by Bonferroni *post hoc* test. *$p<0.05$.

Iodoacetamide and DNFB do not target myofilament proteins

To exclude direct effects of iodoacetamide or DNFB on myofilament proteins, membrane-permeabilized rat cardiomyocytes were measured before and after incubation with both CK-inhibitors. Maximal, submaximal Ca^{2+}-tension and F_{pas} were not changed in the presence of both inhibitors, indicative that both CK-inhibitors do not directly target myofilament protein functions (Table 3). No phosphorylation differences after iodoacetamide treatment in the intact rat cardiomyocytes (Figure 5) were found for the myofilament regulators of Ca^{2+}-sensitivity of force, including cardiac myosin-binding protein C, cardiac troponin I and myosin light chain, and the regulator of diastolic stiffness titin (data not shown). Altogether, these data strongly suggest that the observed CK-inhibition effects on diastolic impairment (Figs. 5 and 6) are due to its effects on myocardial energy reserve and independent of alterations of myofilament proteins.

Table 3. Response of Ca^{2+}-stimulated maximal and submaximal contraction without and with iodoacetamide or DNFB, in left ventricular rat membrane-permeabilized cardiomyocytes at sarcomere length 2.2 µm.

Sample	before incubation	after incubation	N/n
Iodoacetamide (5 mM)			N=3, n=11
32 µM Ca^{2+}-F_{act} (kN/m^2)	22.3±2.7	21.0±2.3	
4.0 µM Ca^{2+}-F_{act} (kN/m^2)	17.1±2.0	16.2±1.8	
F_{pas} (kN/m^2)	1.7±0.2	1.6±0.2	
DNFB (20 µM)			N=3, n=9
32 µM Ca^{2+}-F_{act} (kN/m^2)	20.0±1.5	19.6±1.5	
4.0 µM Ca^{2+}-F_{act} (kN/m^2)	14.0±1.5	13.8±1.5	
F_{pas} (kN/m^2)	1.2±0.2	1.1±0.2	

N, number of rats; n, number of cardiomyocytes. Cardiomyocytes were measured before and after incubation with 5 mM iodoacetamide or 20 µM DNFB, in relaxing solution, for 5 or 30 minutes, respectively. Data was compared using a paired-sampled Student's t-Test. $p<0.05$ was considered significant.

DISCUSSION

Diastolic function is both dependent on the rate of myocardial relaxation and passive properties of the wall that allow proper filling of the ventricle. In this study we provide evidence that synergistic actions of ADP and Ca^{2+} increase actomyosin interactions which elevate myocardial stiffness and limit proper filling of the heart. Our findings show that increased cross-bridge interactions may lead to diastolic dysfunction, in environments with elevated ADP and increased Ca^{2+}, evidenced by high cardiomyocyte stiffness and impaired diastolic re-lengthening, associated with limited ventricular compliance. In human and rat membrane-permeabilized cardiomyocytes,

physiological levels of ADP led to an increased myofilament Ca^{2+}-sensitivity and stiffness, indicating a slowing or incomplete ventricular relaxation with high diastolic stiffness. In support, CK-inactivation limited restoration of diastolic length in intact rat cardiomyocytes and decreased ventricular compliance in whole rat hearts. Altogether these findings indicate that the increased [ADP] as observed in cardiac diseases, in which the metabolic function is compromised, significantly contributes to impaired diastolic function.

Elevated ADP and Ca^{2+} contribute to diastolic dysfunction

Combining studies in human and rat cardiomyocytes and hearts, we show that high ADP and increased Ca^{2+} enhance diastolic stress. In human and rat membrane-permeabilized cardiomyocytes a combination of Ca^{2+} and physiological ADP levels increased Ca^{2+}-sensitivity and cross-bridge stiffness (Figs. 2A and 2D, and Figs. 3A and 3C). Similar findings in the presence of supra-physiological levels of ADP have been shown to elevate Ca^{2+}-sensitivity and sarcomere stiffness in both skeletal rabbit myofibrils[35-37] and rat trabeculae[38]. Additionally, in intact rat cardiomyocytes CK-inhibition limited diastolic relaxation (Fig. 5B). LV compliance also declined in isolated rat hearts (Fig. 6). Similarly, it was previously shown by Tian et al.[9,16] that myocardial ADP accumulation increases LVEDP and impairs myocardial relaxation in isolated rat hearts. However, these authors argued that these changes are Ca^{2+}-independent.[16] Our findings instead support the hypothesis that Ca^{2+} exacerbates the decline in LV compliance in the presence of ADP. This is shown by the observation that buffering Ca^{2+} prevented the decrease in compliance during CK-inactivation (Fig. 6B). Conversely, a low concentration of caffeine (200 μM), to induce diastolic Ca^{2+}-release, showed a modest increase in PV slope compared to CK-inhibition (Fig. 6B). Thus, Ca^{2+} alone cannot completely account for the observed decline of LV compliance. These data suggest that in the absence of ADP resting diastolic Ca^{2+} has a relatively small contribution to diastolic stiffness and that ventricular compliance only declines in the presence of diastolic Ca^{2+} and high ADP caused by an active cross-bridge component. We provide evidence that high ADP and Ca^{2+}-dependent cross-bridge formation imposes high myocardial diastolic stiffness associated with greater than normal filling pressure, which contribute to diastolic dysfunction.

Augmented contractility following elevations of ADP and Ca^{2+}

On the basis of the observed diastolic impairment of intact rat cardiomyocytes we would expect a reduced fractional shortening, resulting from restricted recruitment of the Frank-Starling reserve. This would decrease the preload-induced force of contraction. In contrast, contractility was increased as evidenced by enhanced shortening capacity of intact rat cardiomyocytes following CK-inactivation (Fig. 5E). The greater than normal contractility can be explained by the increased Ca^{2+}-sensitivity of force production induced by physiological levels of ADP (Figs. 2A and 3A) and the Ca^{2+}-overload state promoted by CK-inactivation (Figs. 5F and 5G, and Table 2).

Notably, our analysis reveals that the early inotropic response following CK-inactivation (phase II) is not caused by disturbed Ca^{2+}-transient (Figs. 5F and 5G, and Table 2) and thus explained by the ADP-induced increase of myofilament Ca^{2+}-sensitivity (Figs. 2A and Fig. 3A). However, prolonged CK-inhibition (phase III) still leads to increased contractility, but also significantly impairs diastolic re-lengthening (Fig. 5B), both of which are likely caused by systolic and diastolic Ca^{2+}-overload (Figs. 5F and 5G, and Table 2), respectively. Our results support the theory that CK-inactivation by increases of cytosolic ADP stimulates a Ca^{2+}-overload state. The Ca^{2+}-overload can result from: 1) the high Ca^{2+}-sensitivity promoted by ADP at the myofilaments; because the myofilaments are the main source for the buffering of Ca^{2+} during the cardiac cycle[39], evidence from animal models supports that high myofilament Ca^{2+}-sensitivity increases cytosolic Ca^{2+}-binding with altered Ca^{2+}-homeostasis in animals[40,41]; and/or 2) the high [ADP/ATP] ratio caused by ADP accumulation that reduces the free energy released from ATP hydrolysis (ΔG_{ATP}). Since the sarcoplasmic reticulum Ca^{2+}-ATPase, and other sarcolemmal pumps, including the Na^+/K^+-ATPase, operate close to their theoretical thermodynamic limits, the rise of [ADP] will favor diastolic Ca^{2+} rise.[7] However one cannot exclude that iodoacetamide also has an effect on Ca^{2+}-release via the ryanodine receptor as was shown in skeletal muscle from rabbits.[42] Hence, in our experiments 'artificial' elevation of Ca^{2+} that is not coupled to CK-inhibition may also be present.

Enhanced actomyosin interactions lead to high diastolic stress

It has been proposed that expression of the stiffer titin isoform or its altered phosphorylation represents a major contributor to high diastolic stiffness observed in HF patients with diastolic dysfunction.[3,5] Within these studies, there is no evidence to support a role for altered cross-bridge interaction to high diastolic stiffness.[3,5] In the majority of the studies membrane-permeabilized cardiomyocytes and muscle strips, with thick- and thin-filament extraction by KCl and KI, have been employed to study passive properties of HF myocardium.[3,5] There are however limitations to the latter approaches that need to be addressed: 1) KCl and KI treatment is designed to assess the contribution of collagen to sarcomere stiffness as it removes thick- and thin-filament anchors in the sarcomere, and thereby abolishes any contribution of the actomyosin interaction[43]; 2) chemical digestion of the sarcolemma in membrane-permeabilized cardiomyocytes causes myofilament lattice expansion (and less approximation of myosin towards actin) that decreases force generation[44,45]; 3) the relatively low temperature (15°C) used in the experiments reduces cross-bridge active force, shortening velocity and power[46]; 4) rapid shortening and re-lengthening procedures mechanically detach myosin from actin[47,48]; and importantly 5) membrane-permeabilized cardiomyocytes lack important contributors in the intracellular milieu of normal and failing intact cardiomyocytes. These include the *in vivo* diastolic Ca^{2+} and cytosolic ADP. King et al.[28] has recently reported that cross-bridges account for up to ~30% of diastolic stress at a sarcomere length of 2.1 μm using mouse membrane-

permeabilized cardiomyocytes measured at 37°C in the presence of dextran to normalize lattice spacing. In the present study we adapted this protocol to evaluate the cross-bridge contribution to diastolic stress in rat membrane-permeabilized cardiomyocytes under disease conditions, including those where metabolic function is affected that elevates ADP in the presence of diastolic Ca^{2+}, and showed that cross-bridges greatly contribute to diastolic stress (Fig. 4). Our data support the idea that cross-bridges may slow myocardial relaxation and impose high diastolic stiffness in disease, and in concert with titin and other diastolic mediators, contribute to diastolic dysfunction in HF patients. Importantly our results support the notion that the diastolic phase is not purely a passive process, but is accompanied by active stress.

Limitations

At present, we are unable to directly control intracellular ADP concentration in the intact cardiomyocytes and whole heart preparations. To increase cytosolic ADP, we opted for the use of two well-known drugs that inhibit the CK reaction, i.e. iodoactemide and DNFB. Iodoacetamide allows us to estimate ADP elevations due to its well-characterized effects on the ATP-regeneration system.[16] Based on previous findings using similar amounts of iodoactemide[16], ADP is estimated not to exceed 73 μM in our experiments, while ATP, PCr, inorganic phosphate and pH levels are unaltered. A limitation of iodoactemide which makes it unsuitable for whole heart experiments, is that it unspecifically targets enzymes of the glycolytic reaction.[16] This is not problematic in single cells because of the short duration of the experiment (3 min). In contrast, this might be detrimental in the whole heart experiments, which required longer periods of CK-inhibition (30 min). Therefore, DNFB was used in those experiments, as it does not target enzymes of the glycolytic pathway.[32] However, using DNFB we are unable, to our knowledge, to carefully estimate the expected intracellular ADP concentration. We believe that overall the effects of both drugs are interchangeable between experiments, which is partly supported by similar inhibition of the CK reaction (~40%).

Conclusion

Based on our experimental data, we propose that conditions which elevate myocardial ADP and Ca^{2+} contribute to diastolic dysfunction by increasing residual actomyosin interactions. This leads to high cardiomyocyte stiffness and impaired relaxation, associated with limited ventricular compliance. Our study provides a mechanistic basis for how the elevated ADP and diastolic Ca^{2+}-overload can contribute to diastolic dysfunction in HF patients.

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ADDITIONAL INFORMATION

Competing interests

None

Authors contributions

The objective of our study was to demonstrate that elevations of ADP in the presence of low cytosolic Ca^{2+}, at concentrations present during the diastolic phase *in vivo*, are sufficient to cause diastolic abnormalities. All authors approved the final version of the manuscript. Conception and design of the experiments: V.S. devised, designed and performed the project, analyzed data and wrote the manuscript; A.N. performed experiments, analyzed data and revised the manuscript; M.M. performed experiments, analyzed data and revised the manuscript; E.D.F. performed experiments, analyzed data and revised the manuscript; I.A.E.B. performed experiments, analyzed data and revised the manuscript; R.C.I.W. wrote and revised the manuscript; C.d.R. collected the human cardiac samples and revised manuscript; M.H. analyzed data and revised the manuscript; E.W. wrote and revised the manuscript; G.J.M.S. supervised the project and revised the manuscript; J.T. wrote and revised the manuscript; D.W.D.K. supervised the project, wrote and revised the manuscript; J.v.d.V. supervised the project, wrote and revised the manuscript.

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Translational Perspective

Here we show that actomyosin-related force development may be an important contributor to high diastolic stiffness and may explain the development of diastolic dysfunction in environments where high ADP and increased diastolic [Ca^{2+}] are present, such as in the failing myocardium. These findings establish a clear link between deficits of myocardial energy reserve and disturbed Ca^{2+}-handling, which together leads to inappropriate cross-bridge formation and high diastolic stiffness. These findings may provide insight in how energy-sparing therapies (that reduce cellular ATP usage), such as beta-blockers, angiotensin-receptor blockers and angiotensin converting enzyme inhibitors, improve symptoms and reduce mortality in congestive HF patients [1]. Our data highlight the need for more targeted therapies aimed at lowering myocardial ADP levels to improve the prognosis of human HF.

Table 2. Cardiomyocyte shortening and Ca^{2+}-transient in rat cardiomyocytes with and without iodoacetamide.

	Phase I		Phase II		Phase III	
	Ctrl-group	**IA-group**	**Ctrl-group**	**IA-group**	**Ctrl-group**	**IA-group**
Shortening						
Diastolic SL (µm)	1.85±0.01	1.85±0.01	1.85±0.01	1.83±0.01§	1.85±0.01	1.81±0.01‡#
Shortening velocity (µm/s)	-4.9±0.3	-4.5±0.4	-5.0±0.2	-5.1±0.3	-5.2±0.3	-5.3±0.3‡
Time to peak shortening (s)	0.060±0.004	0.060±0.003	0.061±0.004	0.065±0.003§	0.061±0.004	0.066±0.003‡
Sarcomere shortening (%)	8.2±0.5	7.3±0.5	8.3±0.4	9.0±0.6§	8.7±0.4	9.4±0.6‡
Systolic SL (µm)	1.70±0.01	1.71±0.02	1.70±0.01	1.66±0.02§	1.69±0.01	1.63±0.01‡#
Relaxation velocity (µm/s)	3.5±0.2	3.2±0.3	3.6±0.2	3.5±0.3	3.7±0.2	3.6±0.3
Time to 90% relaxation (s)	0.15±0.01	0.17±0.01	0.15±0.01	0.17±0.01	0.15±0.01	0.21±0.03
Ca^{2+}-transient						
Diastolic Ca^{2+} (F340/380)	1.00±0.09	0.98±0.08	0.99±0.09	1.05±0.08	0.96±0.08	1.17±0.08‡
Time to peak Ca^{2+} release (s)	0.028±0.002	0.028±0.001	0.028±0.001	0.031±0.002	0.030±0.001	0.036±0.003‡
Systolic Ca^{2+} (F340/380)	1.58±0.15	1.57±0.14	1.51±0.15§	1.54±0.15	1.39±0.12‡	1.56±0.12
Ca^{2+} amplitude (F340/380)	0.58±0.08	0.60±0.07	0.49±0.07§	0.49±0.07§	0.42±0.06‡#	0.38±0.06‡#
Tau (s)	0.095±0.005	0.094±0.006	0.094±0.005	0.096±0.007	0.096±0.007	0.132±0.015‡#

Ctrl, Control; IA, Iodoacetamide; SL, sarcomere length. Data was compared using repeated measures 1-way ANOVA followed by Bonferroni *post hoc* test. $p<0.05$ was considered significant; §1-way ANOVA *post hoc* test phase I versus corresponding phase II; ‡1-way ANOVA *post hoc* test phase I versus corresponding phase III; #1-way ANOVA *post hoc* test phase II versus corresponding phase III; 7 heart samples from adult wild-type male Wistar rats were used; 17 to 18 (Ctrl-group) and 17 to 20 (IA-group) cardiomyocytes were analysed.

6

ADP-STIMULATED CONTRACTION: A PREDICTOR OF THIN-FILAMENT ACTIVATION IN CARDIAC DISEASE

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ADP-stimulated contraction:
a predictor of thin-filament activation in cardiac disease

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*"Nature, and Nature's Laws lay hid in Night. God said, Let Newton be!
and All was Light."*

Alexander Pope

ABSTRACT

Diastolic dysfunction is general to all idiopathic dilated (IDCM) and hypertrophic cardiomyopathy (HCM) patients. Relaxation deficits may result from increased actin-myosin formation during diastole due to altered tropomyosin position, which block myosin-binding to actin in the absence of Ca^{2+}. We investigated if ADP-stimulated force development (without Ca^{2+}) can be used to reveal changes in actin-myosin blockade in human cardiomyopathy cardiomyocytes. Cardiac samples from HCM patients, harboring thick- (*MYH7*$_{mut}$, *MYBPC3*$_{mut}$) and thin-filament (*TNNT2*$_{mut}$, *TNNI3*$_{mut}$) mutations, and IDCM, were compared with sarcomere mutation-negative HCM (HCM$_{smn}$) and non-failing donors. Myofilament ADP-sensitivity was higher in IDCM and HCM compared with donors, while it was lower for *MYBPC3*. Increased ADP-sensitivity in IDCM, HCM$_{smn}$ and *MYH7*$_{mut}$ was caused by low phosphorylation of myofilament proteins, as it was normalized to donors by protein kinase A (PKA) treatment. Troponin exchange experiments in a *TNNT2*$_{mut}$ sample corrected the abnormal actin-myosin blockade. In *MYBPC3*$_{trunc}$ samples, ADP-sensitivity highly correlated with cardiac myosin-binding protein-C (cMyBP-C) protein level. Incubation of cardiomyocytes with cMyBP-C antibody against the actin-binding N-terminal region reduced ADP-sensitivity, indicative of cMyBP-C's role in actin-myosin regulation. In the presence of Ca^{2+}, ADP increased myofilament force development and sarcomere stiffness. Enhanced sarcomere stiffness in sarcomere mutation-positive HCM samples was irrespective of the phosphorylation background. In conclusion, ADP-stimulated contraction can be used as a tool to study how protein phosphorylation and mutant proteins alter accessibility of myosin-binding on actin. In the presence of Ca^{2+}, pathologic [ADP] and low PKA-phosphorylation, high actin-myosin formation could contribute to the impaired myocardial relaxation observed in human cardiomyopathy.

SIGNIFICANT STATEMENT

Diastolic dysfunction is characteristic of patients with cardiomyopathy. Evidence indicates that diseased hearts show basal sarcomeric activation capable of impairing diastolic performance. By activating human cardiomyopathy muscle in ADP-containing solutions without Ca^{2+} we showed that actin-myosin blockade is disrupted. This may be caused by the presence of mutations and/or the reduced phosphorylation of myofilament proteins. Our mechanistic study supports the novel idea that protein kinase A-target phosphorylation and myosin-binding protein-C regulate the OFF-ON transition of the thin-filaments. ADP increased myofilament force and stiffness in the presence of Ca^{2+} in cardiomyopathy samples, suggesting this condition limits muscle relaxation through increased actin-myosin interactions. We conclude that ADP-stimulated contraction can be used to reveal conformational changes in the 3-state model of thin-filament activation.

INTRODUCTION

Heart failure (HF) is a syndrome clinically defined as the inability of the heart to sufficiently supply blood to organs and tissues.[1] Systolic dysfunction is present in approximately half of the HF population, while diastolic dysfunction is a common feature in almost all HF patients.[2] Moreover, in hypertrophic cardiomyopathy (HCM), which is caused by mutations in genes encoding thin- and thick filament proteins, impaired diastolic function is frequently observed.[3] Impaired relaxation of the heart may be caused by high myofilament Ca^{2+}-sensitivity. This increased sensitivity for Ca^{2+} would result in residual myofilament activation at diastolic [Ca^{2+}], which may delay the onset of ventricular relaxation and limit proper filling of the heart. High myofilament Ca^{2+}-sensitivity has been observed in both acquired and genetic forms of cardiomyopathy.[3,4] In human idiopathic dilated cardiomyopathy (IDCM), high myofilament Ca^{2+}-sensitivity has been associated with reduced β-adrenergic receptor-mediated phosphorylation by protein kinase A (PKA).[4] Reduced PKA-phosphorylation of cardiac troponin I (cTnI) and cardiac myosin-binding protein-C (cMyBP-C) increases myofilament Ca^{2+}-sensitivity.[5-8] Likewise, high myofilament Ca^{2+}-sensitivity is a common characteristic of HCM and may be caused by the mutant protein or by reduced PKA-mediated protein phosphorylation secondary to HCM disease progression.[3,9]

Contractile performance of the heart muscle may thus be perturbed by mutation-induced and phosphorylation-mediated protein changes that affect thin-filament transitions. Ca^{2+}-induced cardiac muscle contraction is tightly modulated by the troponin-tropomyosin complex that regulates the interactions between the actin thin-filament and myosin thick-filament (i.e. cross-bridge formation). Accordingly, the myofilaments oscillate between three transitions termed the blocked (B-state), closed (C-state) and open (M-state) states of thin-filament regulation that represent the distinct position of tropomyosin on actin(Fig. 1).[10-12] In the absence of Ca^{2+} (B-state), tropomyosin sterically blocks the myosin-binding sites on actin (Fig. 1A). Upon electrical activation of cardiomyocytes the rise of cytosolic [Ca^{2+}] alters the conformation of the troponin-tropomyosin complex, which moves tropomyosin on actin and exposes myosin-binding sites (C-state). Weakly-bound cross-bridges (myosin-ADP-Pi) populate the C-state (Fig. 1B).[10,12] Transition to the M-state involves release of inorganic phosphate (Pi) from the cross-bridge and strong-binding cross-bridge formation (myosin-ADP) that induces additional movement of tropomyosin, resulting in myofilament contraction and sliding (Fig. 1C).

The 3-state model of cross-bridge interaction implies that the main task of Ca^{2+} is to uncover myosin-binding sites on actin and that formation of myosin-ADP represents the main regulator of force development and contraction. Notably, solution[10] and cryo-electron microscopy[13] studies have shown that in the absence of Ca^{2+} the myofilaments are not entirely blocked, as ~5% of the thin-filaments have tropomyosin localized in the C-state position. This observation suggests that conditions that promote myosin-ADP formation can trigger myofilament contraction in Ca^{2+}-free conditions and thereby impair relaxation. Indeed, in membrane-permeabilized rabbit

skeletal muscle fibers[14], bovine myocardium[15,16] and human cardiac muscle[17] mM levels of ADP stimulate force development in the absence of Ca^{2+}.

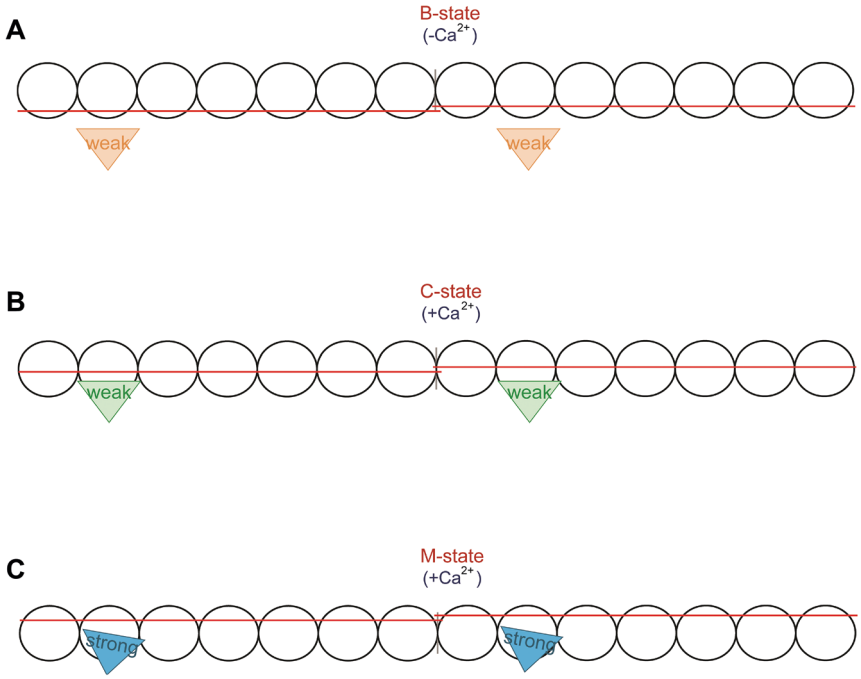

Figure 1. Three-state model of thin-filament activation. Seven actin monomers (circles), spanned by one tropomyosin dimer (red strand) together with the troponin complex (not shown) comprise one functional unit (A_7TmTn). Two functional units are depicted, and individual myosins are shown as triangles (weak, weak-binding cross-bridges; strong, strong-binding cross-bridges). (A) B-state (blocked); when ATP is present and cytoplasmic [Ca^{2+}] is low and is not bound to cardiac troponin C (cTnC), tropomyosin is sterically blocking the myosin-binding sites on actin. (B) C-state (Ca^{2+}-induced); upon rise in cytoplasmic [Ca^{2+}], Ca^{2+} binds to cTnC, inducing conformational changes of the troponin complex, resulting in a ~25° movement of tropomyosin on the thin-filament, thereby exposing myosin-binding sites on actin. In the C-state the myofilament is not yet activated as non-tension-generating cross-bridges bind weakly to actin. (C) M-state (Myosin-induced); the strong-binding of tension-generating cross-bridges induces a ~10° movement of tropomyosin on actin, resulting in myofilament activation and contraction.

Since ADP-stimulated contraction is due to myosin-ADP binding to the non-blocked sites of the thin-filament in the absence of Ca^{2+}, it provides an experimental tool to assess changes in tropomyosin's position in acquired and genetic cardiomyopathies in which altered protein phosphorylation and mutant proteins may alter myofilament activation. In addition, it could represent a pathomechanism underlying the diastolic dysfunction seen in both disease states. Solution studies with mutant troponin proteins, which are known to cause HCM, showed a reduction in the B-state at low

Ca^{2+} conditions compared to wild-type troponin proteins.[18,19] Mutation-induced irregularities in troponin-tropomyosin interactions disrupt the B-state and shift the thin-filament to the C-state, increasing the available myosin-binding sites on actin.

In addition to Ca^{2+}-induced changes of the thin-filament, tropomyosin location may also be altered by the thick-filament protein cMyBP-C. Recent evidence supports that the N-terminal extension of cMyBP-C binds the low Ca^{2+}-state (B-state) position of tropomyosin on actin and interferes with tropomyosin-actin interactions, dislocating tropomyosin into the C-state position (i.e. the presence of cMyBP-C sensitizes the thin-filament to Ca^{2+}).[20,21] Since it was previously shown that in Ca^{2+}-free conditions (B-state) approximately 5% of the thin-filaments (lacking cMyBP-C) have tropomyosin localized in the C-state position[10], more myofilaments may be in the C-state in the presence of cMyBP-C. We[22] and others[23] have shown that cMyBP-C mutations, that are a major cause of HCM, have a reduced level of healthy cMyBP-C protein compared to non-failing hearts (i.e. haploinsufficiency), which may alter tropomyosin position on the thin-filament.

To verify if ADP-stimulated contraction provides an experimental tool to assess mutation-induced and phosphorylation-mediated changes in thin-filament transitions, that precede Ca^{2+}-activation of myofilaments, we tested the following hypotheses: 1) that IDCM and HCM samples with thin-filament mutations are more sensitive to ADP, as a result of a higher accessibility of myosin-binding sites on actin, while 2) cMyBP-C haploinsufficient HCM myocardium has a reduced ADP-sensitivity (i.e. less cMyBP-C causes reduced displacement of tropomyosin from the B-state) compared with cells from non-failing hearts. To answer our hypotheses we activated membrane-permeabilized human cardiomyocytes in ADP containing Ca^{2+}-free solutions. Cells were isolated from HCM patients with mutations in genes encoding thick- (*MYH7*, *MYBPC3*) and thin-filament (*TNNT2, TNNI3*) proteins and patients with IDCM and compared with cells from sarcomere mutation-negative HCM (HCM_{smn}) and non-failing donors. Finally, we investigated if the ADP level as observed in diseased hearts, in the presence of Ca^{2+}, increases myofilament force development in cardiomyocytes from human cardiomyopathy hearts.

We conclude that in HCM with thin-filament mutations tropomyosin's ability to block myosin-binding sites on actin is reduced. This effect is exacerbated in HCM samples by the low PKA-phosphorylation of myofilament proteins, which is also observed in human IDCM. In contrast, cMyBP-C HCM-causing mutations reduce accessibility of myosin for actin. The findings in this study provide evidence that ADP-mediated activation can be used as an experimental tool to reveal mutation- and phosphorylation-mediated changes in tropomyosin location on the thin-filament.

METHODS

Myocardial human samples

Human left ventricular (LV) tissue was obtained during open heart surgery from end-stage failing (n=4; IDCM) and HCM patients. HCM heart samples harboring thick- and thin-filament gene mutations were obtained during myectomy surgery to relieve LV outflow obstruction. Our study included patients carrying heterozygous mutations in *MYBPC3* (n=13), *MYH7* (n=3; $MYH7_{mut}$) and *TNNI3* (n=1; $TNNI3_{mut}$) and 5 HCM patients in whom no mutation was found after screening of 8 genes (sarcomere mutation-negative HCM; HCM_{smn}). The *MYBPC3* group consisted of patients with truncating (n=10; $MYBPC3_{trunc}$) and missense (n=3; $MYBPC3_{mut}$) mutations. Data for these two *MYBPC3* groups are presented separately. Tissue was also obtained from 1 end-stage failing HCM patient carrying a homozygous *TNNT2* mutation ($TNNT2_{mut}$). Specific gene mutation information of HCM patients is listed in Table 1. Heart tissue from 4 non-failing donors served as controls. IDCM and non-failing donor samples were collected in cardioplegic solution. Tissue was immediately frozen and stored in liquid nitrogen. Samples were obtained after written informed consent and the study protocol was approved by the local ethical committees. The investigation conforms with the principles outlined in the Declaration of Helsinki (1997).

Experimental solutions for isometric force measurements in membrane-permeabilized cardiomyocytes

The composition of all solutions was calculated based on a computer program similar to that described previously.[24] The pH of all solutions was adjusted to 7.1 at 15°C by KOH and ionic strength was adjusted to 180 mM with KCl. The relaxing solution contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and 14.5 mM phosphocreatine (PCr). ADP-activating solutions contained 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES and MgADP in increasing concentrations from 1 to 10 mM. Nominal free Ca^{2+} was present. Ca^{2+}- activating solutions in the presence of 100 µM ADP consisted of 2 mM free Mg^{2+}, 1 mM MgATP, 20 mM EGTA, 10 mM BES, and free Ca^{2+} varied from 1 to 32 µM. PCr was omitted from the activating solutions to preserve the MgADP/MgATP ratio. All force measurements were performed at 15°C. We maintained [ATP] constant at 1 mM to avoid rigor cross-bridge formation (reported to occur when [ATP] falls below 0.1 mM[25]).

Isometric force measurements

Small human cardiac tissue samples were thawed in relaxing solution and cardiomyocytes were mechanically isolated by tissue disruption as described previously.[26] Cardiomyocytes were membrane-permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a piezoelectric motor. The sarcomere length of the preparation was measured in relaxing solution and adjusted to 2.2 µm. Average sarcomere lengths

were determined by means of a spatial Fourier transformation as described previously.[26] The force measurements started with a maximal activation at ADP concentration of 10 mM (ADP-F_{total}). After a series of seven activations at submaximal ADP concentrations, another measurement was carried out at maximal ADP activation of 10 mM. Total ADP-force was measured from the zero force value obtained from slacking the cardiomyocyte to 70% of its original length within 1 ms, after the plateau of force was reached. Force was corrected for myocyte deterioration by linear interpolation between maximal force values. The intermediate results, obtained at higher ADP values, were normalized to the interpolated maximal force values. The measurements were continued until a full ADP-force curve was obtained. Cells were excluded from the study when the final force measurement at maximal ADP was less than 80% compared to the first maximal control measurement. To obtain passive force (F_{pass}) the cardiomyocyte was slackened to 70% of its original length for 10 seconds in relaxing solution by means of the piezoelectric motor from the initial sarcomere length of 2.2 µm. The decrease in force during this procedure equals the passive force at 2.2 µm sarcomere length. Active tension (ADP-F_{act}) was calculated as $F_{act}=F_{total}-F_{pass}$. Absolute tensions were normalized to cardiomyocyte cross-sectional area and expressed as developed tension (in kN/m^2). Similarly, Ca^{2+}-force measurements in the presence of 100 µM ADP were determined as described previously.[17] Residual force measurements, an indication of sarcomere stiffness, were measured after a slack-restretch procedure at high Ca^{2+} (32 µM) with 100 µM ADP as follows: after steady state force was reached, cardiomyocytes were shortened to 70% of their original length within 1 ms and then re-stretched to the original length after 30 ms. Residual force was calculated from the initial force recovery reached after the length change and normalized to each total steady-state force reached before length change. Measurements are normalized to each corresponding total force as described previously.[17] Finally, force measurements were performed following exogenous protein kinase A (PKA) treatment of cells for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U/incubation, Sigma). To control for protocol artefacts (e.g. incubation time, buffer solution) cardiomyocytes harvested from failing hearts were incubated with PKA-buffer solution but without PKA. No statistical changes were observed between the absence (3.59±0.17 mM ADP) and presence of PKA-buffer (3.65±0.20 mM ADP).

Data analysis isometric force measurements

Ca^{2+}- and/or ADP-force relations were determined by a non-linear fit procedure using a modified Hill equation using KaleidaGraph version 3.6 (Synergy Software, Reading, PA) as follows:

$$P(\text{ADP}) / P_0 = [\text{ADP}]^{nH} / (K^{nH} + [\text{ADP}]^{nH})$$

$$P(Ca^{2+}\text{-ADP}) / P_0 = [Ca^{2+}\text{-ADP}]^{nH} / (K^{nH} + [Ca^{2+}\text{-ADP}]^{nH})$$

where *P* is steady-state force. P_0 denotes the steady isometric force at either high [ADP] and saturating [Ca^{2+}] with ADP, *nH* describes the steepness of the relationship, and K represents the [Ca^{2+}] and/or [ADP] at which force is half-maximal (0.5 x P_0). Myofilament Ca^{2+} and/or ADP-sensitivity is denoted as EC_{50}.

Exchange of recombinant human wild-type troponin complex in single cardiomyocytes

Troponin exchange protocol

Troponin exchange was performed as described previously.[8,9] In brief, single cardiomyocytes from the $TNNT2_{mut}$ heart were mechanically isolated by tissue disruption in ice-cold rigor solution (132 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris,

Table 1. Hypertrophic cardiomyopathy samples information.

Samples		Mutation	Type
$MYBPC3_{trunc}$	Cardiac myosin-binding protein-C (cMyBP-C)		Truncating mutations
1		p.Y340X	termination codon
2		p.R493X	termination codon
3		c.927-2A>G	splice site
4		c.927-2A>G	splice site
5		c.1458-1G>C	splice site
6		c.2373duplicationG	insertion
7		c.2373duplicationG	insertion
8		c.2373duplicationG	insertion
9		c.2864.2865delCT	deletion
10		c.3407.3409del	deletion
$MYBPC3_{mut}$	Cardiac myosin-binding protein-C (cMyBP-C)		Missense mutations
11		p.E258K	missense
12		p.G531R	missense
13		p.R597Q	missense
$MYH7_{mut}$	Myosin-heavy chain (MyHC)		
14		p.R403Q	missense
15		p.R403Q	missense
16		p.V606M	missense
$TNNT2_{mut}$	Cardiac troponin T (cTnT)		
17		p.K280N	missense
$TNNI3_{mut}$	Cardiac troponin I (cTnI)		
18		p.R145W	missense

trunc, truncating mutation; mut, missense mutation; p, protein amino residue; c, codon region; X, stop codon.

5 mM EGTA, 1 mM NaAzide, pH 7.1). Cardiomyocytes were membrane-permeabilized by incubation for 5 minutes in rigor solution containing 0.5% (v/v) Triton-X100. After permeabilization, cardiomyocytes were washed twice with rigor solution followed by washing in exchange solution (10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, pH 6.9). Next, single cardiomyocytes were incubated overnight at 4°C in exchange solution containing the appropriate concentration of recombinant human wild-type troponin complex (1.0 mg/mL) with the addition of 4 mM $CaCl_2$, 4 mM DTT, 5 µl/mL protease inhibitor cocktail (PIC, Sigma, P8340) and 10 µl/mL phosphatase inhibitor cocktail 2 and 3 (PhIC, Sigma, P2850, P5726) (pH 6.9). The next day, cells were washed twice in rigor solution followed by washing in relaxing solution.

Determination of troponin exchange percentage

Half of the cardiomyocyte suspension was used for isometric force measurements, while the other half was used to analyze troponin exchange percentage. This half was treated with 2D-clean-up kit (GE Healthcare), homogenized in sample buffer (15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT) and protein concentration was measured with *RCDC* Protein Assay kit II (Biorad).

To determine the degree of exchange of endogenous mutant troponin by recombinant wild-type cardiac troponin Western blotting was performed. Recombinant wild-type cTnT contains a myc-tag, which allowed differentiation between endogenous and recombinant cardiac troponin complex. Proteins were separated on a 1D SDS-PAGE and blotted onto a nitrocellulose membrane. A specific monoclonal antibody was used against cTnT (Clone JLT-12, Sigma) to detect endogenous and recombinant cTnT by chemiluminescence (ECL, Amersham Biosciences) as described previously.

Myofilament protein phosphorylation and levels

Phosphorylation of cTnI

ProQ-Diamond stained 1D-gels were used to assess phosphorylation of the myofilament protein cTnI as previously described.[27]

Western blot analysis of cMyBP-C total protein

Total protein of cMyBP-C was assessed using specific antibodies in Western blots (E-7; sc-137180, Santa Cruz Biotechnology).

Incubation of failing cardiomyocytes with specific cMyBP-C antibodies

Single cardiomyocytes from 4 IDCM samples were incubated for 90 minutes with a 1:100 dilution of either specific N' cMyBP-C antibody (mouse monoclonal E-7; sc-137180, Santa Cruz Biotechnology), C' cMyBP-C antibody (generously provided by Sakthivel Sadayappan, Loyola University Chicago, Maywood, IL, USA) or an antibody against nucleolar protein nucleophosmin (mouse monoclonal, sc-32256, Santa Cruz

Biotechnology). The C' cMyBP-C antibody or nucleolar phosphoprotein antibody (control ab) served as the incubation negative controls.

Statistical analysis

Data analysis and statistics were performed using Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA) and SPSS version 15.0 (IBM, Armonk, NY). Data are presented as mean ± SEM of all single cardiomyocytes per patient group (8 groups, i.e. 1 IDCM group, the 5 HCM sarcomere mutation positive groups (HCM_{mut}), HCM_{smn} and non-failing donor). To take into account the repeated sample assessments within patient/donor groups multilevel analysis was performed as shown previously.[9] Comparison between all groups was performed for ADP-sensitivity and Ca^{2+}-ADP-sensitivity at 2.2 µm sarcomere length before and after PKA treatment. All data was tested for normality using the Shapiro-Wilk Test. Normality was assumed when $p>0.05$ and the variances were equal. Paired samples t-Test was conducted when one variable was tested between 2 groups of data. When more than one variable was tested in more than 2 groups a 2-way ANOVA was conducted followed by a Bonferroni *post-hoc* test if significant differences were found. Each test is clearly specified in Tables or Figure legends. The level of significance was set at $p<0.05$.

RESULTS

ADP-sensitivity in idiopathic dilated and hypertrophic cardiomyopathy

To evaluate the accessibility of myosin-binding sites on actin, force production of single membrane-permeabilized cardiomyocytes was measured at varying ADP concentrations, in the absence of Ca^{2+}. Tissue from human IDCM and HCM hearts were selected and compared with non-failing donors. Typical tension tracings of single cardiomyocytes from IDCM, $MYH7_{mut}$, $MYBPC3_{trunc}$, and $TNNT2_{mut}$ samples at 2.2 µm sarcomere length during ADP-maximal (10 mM) and submaximal (4 mM) activation are shown in Figure 2.

We have previously shown that all IDCM and HCM samples, except for the HCM $TNNT2_{mut}$ sample, have lower phosphorylation of PKA-target proteins in the myofilament compared to donor myocardium.[4,9] Figure 3*A* shows that the phosphorylation level of the PKA target protein cTnI is lower in the IDCM and HCM samples relative to non-failing donor, which was set to 1. One exception is the homozygous $TNNT2_{mut}$ sample, which showed similar cTnI phosphorylation as in donor myocardium. To determine the effect of low myofilament phosphorylation, force-ADP relations were constructed for all samples before and after PKA treatment (Fig. 2 right panels). As shown in Figure 3*B*, ADP-sensitivity (denoted as EC_{50}) was higher in IDCM and several HCM patient groups, including $MYH7_{mut}$, $TNNT2_{mut}$, $TNNI3_{mut}$ and HCM_{smn}, while it was lower in $MYBPC3_{mut}$ (missense mutations) and $MYBPC3_{trunc}$ (truncating mutations) compared with donors (Table 2). PKA normalized ADP-sensitivity to donor levels in IDCM, $MYH7_{mut}$ and HCM_{smn}, while $TNNT2_{mut}$ and $TNNI3_{mut}$ remained more sensitive

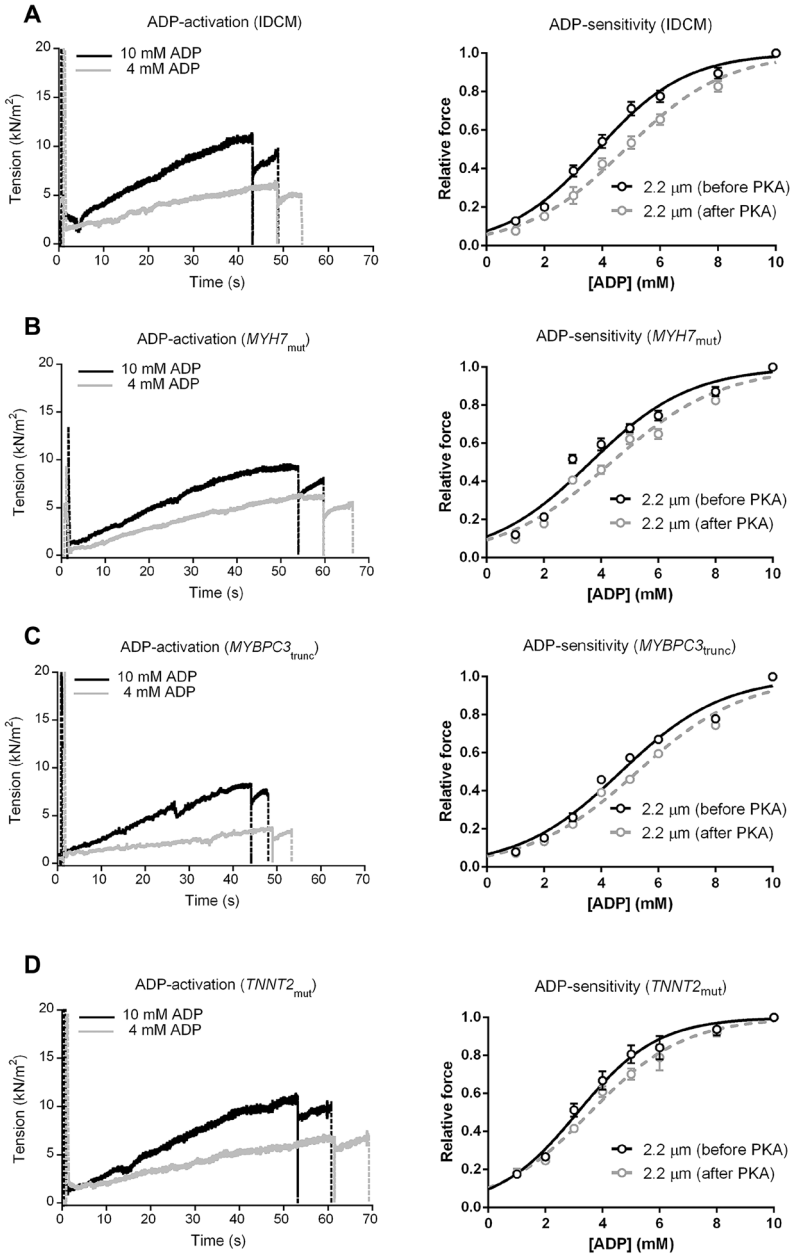

Figure 2. Myofilament ADP-activation and ADP-sensitivity in idiopathic dilated (IDCM) and hypertrophic (HCM) cardiomyopathy membrane-permeabilized cardiomyocytes. Left panels show typical tension recordings of a single membrane-permeabilized cardiomyocytes at 2.2 µm sarcomere length during ADP maximal (10 mM) and submaximal activation (4 mM) at 15°C and 1 mM ATP for IDCM (*A*), *MYH7*$_{mut}$ (B), *MYBPC3*$_{trunc}$ (C) and *TNNT2*$_{mut}$ (D). Right panels show normalized force-ADP relationship for IDCM (A), *MYH7*$_{mut}$ (B), *MYBPC3*$_{trunc}$ (C) and *TNNT2*$_{mut}$ (D) cardiomyocytes before and after protein kinase A (PKA)-treatment. *MYH7*$_{mut}$, myosin heavy chain mutations; *MYBPC3*$_{trunc}$, myosin-binding protein-C (truncating) mutations; *TNNT2*$_{mut}$, cardiac troponin T mutation.

to ADP (Fig. 3C and Table 2). Interestingly, after PKA *MYBPC3*$_{mut}$ and *MYBPC3*$_{trunc}$ were still less sensitive to ADP than donor cells (Fig. 3C and Table 2).

These results indicate that PKA-target phosphorylation of the myofilaments reduces accessibility of myosin-binding sites on actin in the absence of Ca^{2+}. At baseline (before PKA administration) the myofilaments from cardiomyopathy samples are more sensitive to ADP-activation, except for the low sensitivity to ADP of *MYBPC3*$_{mut}$ and *MYBPC3*$_{trunc}$. PKA-phosphorylation normalized sensitivity to ADP in IDCM, *MYH7*$_{mut}$ and HCM$_{smn}$, suggesting that any putative disruption of tropomyosin's B-state position (and the greater accessibility of myosin-binding sites on actin) in these samples may be due to the low baseline phosphorylation of PKA target proteins.

Table 2. ADP-sensitivity (EC_{50}) of non-treated (A) and protein kinase A (PKA)-treated (B) cardiomyocytes.

Samples				
A	**vs. donor**	**p value**	**95% CI (mmol/L)**	**ns**
	IDCM	*p=0.041	-0.83 to -0.018	N=4, n=14
	MYH7$_{mut}$	*p=0.003	-1.08 to -0.23	N=3, n=11
	MYBPC3$_{mut}$ (missense)	*p=0.010	0.14 to 0.98	N=3, n=12
	MYBPC3$_{trunc}$ (truncating)	*p=0.004	0.16 to 0.85	N=10, n=28
	TNNT2$_{mut}$	*p=0.002	-1.57 to -0.36	N=1, n=4
	TNNI3$_{mut}$	*p=0.003	-1.54 to -0.32	N=1, n=4
	HCM$_{smn}$	*p=0.038	-0.77 to 0.02	N=5, n=18
B.	**vs. donor**	**p value**	**95% CI (mmol/L)**	**ns**
	IDCM	p=0.895	-0.44 to 0.39	N=4, n=14
	MYH7$_{mut}$	p=0.106	-0.80 to -0.08	N=3, n=11
	MYBPC3$_{mut}$ (missense)	*p=0.032	-0.04 to 0.92	N=3, n=12
	MYBPC3$_{trunc}$ (truncating)	*p=0.001	0.26 to 0.96	N=10, n=28
	TNNT2$_{mut}$	*p=0.001	-1.67 to -0.41	N=1, n=4
	TNNI3$_{mut}$	*p=0.017	-1.40 to -0.14	N=1, n=4
	HCM$_{smn}$	p=0.870	-0.35 to 0.42	N=5, n=18

Multilevel analysis. *p<0.05 was considered significant. N= number of samples; n= number of cardiomyocytes. 16 cardiomyocytes from 4 non-failing hearts (donor) served as controls; IDCM, idiopathic dilated cardiomyopathy; *MYH7*$_{mut}$, myosin-heavy chain mutations; *MYBPC3*, myosin-binding protein C mutations; *TNNT2*$_{mut}$, cardiac troponin T mutation; *TNNI3*$_{mut}$, cardiac troponin I mutation; HCM$_{smn}$, sarcomere mutation-negative.

cMyBP-C regulates ADP-sensitivity

We[22] and others[23] have shown that cMyBP-C truncating mutations lack the presence of the smaller truncated protein, but have a lower total level of healthy full length cMyBP-C (i.e. haploinsufficiency). To assess if the expression level of cMyBP-C in *MYBPC3*$_{trunc}$ correlates with ADP-sensitivity (as cMyBP-C modulates thin-filament transitions[20,21]), cMyBP-C expression was determined. Total cMyBP-C protein level

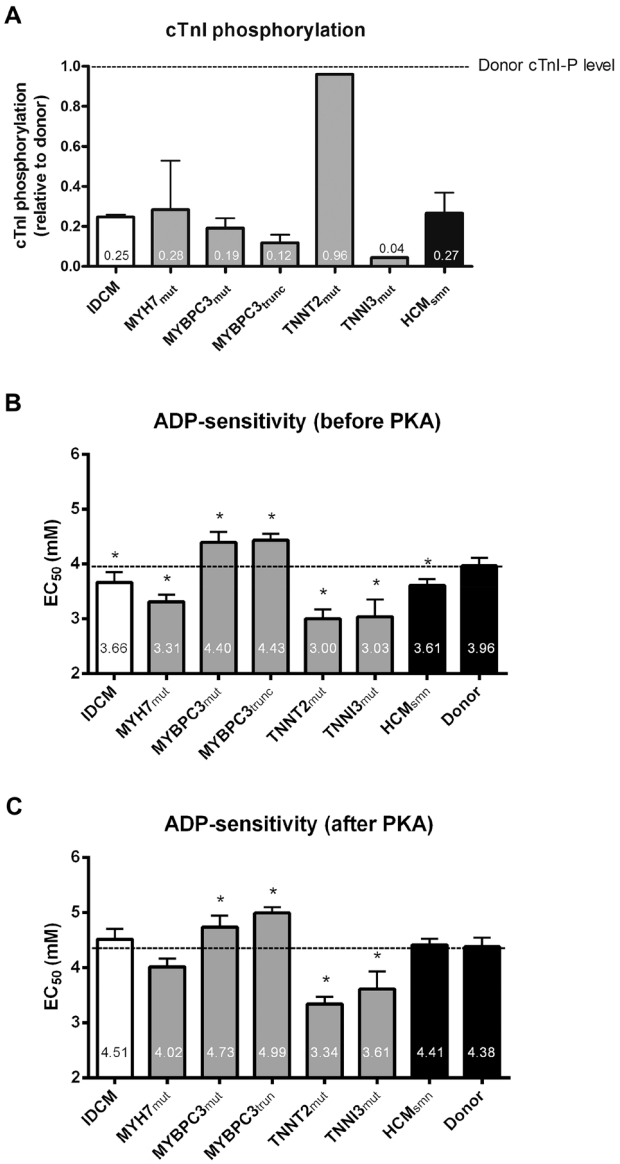

Figure 3. (A) Idiopathic dilated (IDCM) and hypertrophic (HCM) cardiomyopathy samples showed reduced phosphorylation of the protein kinase A (PKA) target protein cardiac troponin I (cTnI) relative to non-failing donor, which was set to 1. Only the homozygous *TNNT2*$_{mut}$ sample showed relatively high cTnI phosphorylation similar as observed in donor tissue. Myofilament ADP-sensitivity in IDCM and HCM cardiomyopathy membrane-permeabilized cardiomyocytes. ADP-sensitivity (EC_{50}) before (B) and after (C) PKA treatment in IDCM, HCM and donor samples. Data was compared using multilevel analysis. Detailed statistics, sample numbers and number of cardiomyocytes used are shown in Table 2. *MYH7*$_{mut}$, myosin heavy chain mutations; *MYBPC3*$_{mut}$, myosin-binding protein-C (missense) mutations; *MYBPC3*$_{trunc}$, myosin-binding protein-C (truncating) mutations; *TNNT2*$_{mut}$, cardiac troponin T mutation; *TNNI3*$_{mut}$, cardiac troponin I mutation; HCM$_{smn,}$ sarcomere mutation-negative HCM samples.

(relative to tropomyosin) was 45±9% reduced in *MYBPC3*$_{trunc}$ compared to controls (Fig. 4A). To correlate cMyBP-C level to thin-filament activation, the level of cMyBP-C per sample was plotted against its ADP-sensitivity value obtained after PKA treatment (to normalize for differences in phosphorylation). Indeed, we observed a strong correlation between cMyBP-C protein level and ADP-sensitivity (Fig. 4*B*), indicating that a reduction in cMyBP-C diminishes the number of available myosin-binding sites on actin (i.e. favors the B-state formation).

To confirm and provide the proof-of-concept that cMyBP-C indeed regulates thin-filament transitions via its N-terminal region, IDCM samples were incubated for 90 minutes with an antibody that binds the C0 domain of cMyBP-C (the domain involved in thin-filament binding). We expected that antibody binding to cMyBP-C, decreased cMyBP-C affinity for the thin-filament. ADP-sensitivity was reduced after incubation with specific N-terminal cMyBP-C antibody, suggesting that antibody competition for cMyBP-C resulted in less cMyBP-C affinity for the thin-filament and hence, a reduced accessibility of myosin-binding sites (Fig. 4*C*). Maximal ADP-F_{act} significantly reduced upon cMyBP-C N-terminal antibody incubation, while it did not affect passive tension (ADP-F_{pass}) (Table 3). To control for protocol artefacts (e.g. unspecific antibody binding), another set of cardiomyocytes was incubated for 90 minutes with an antibody against the C10 domain of cMyBP-C (domain involved in cMyBP-C anchoring to the thick-filament) or a control antibody (against nucleophosmin, a nucleolar protein). Increased responsiveness to ADP was found for the C10 antibody associated with a small drop in ADP-F_{pass}, while no alterations in ADP-sensitivity, maximal force and ADP-F_{pass} was found using nucleolar protein antibody (Fig. 4*C* and Table 3). These data support that the effects seen for the C0 antibody were specific to the N-terminal region of cMyBP-C.

Finally, haploinsufficiency was also reported in HCM with *MYBPC3* missense mutations[23], including the mutation investigated in our study (E258K). Indeed, samples with *MYBPC3*$_{mut}$ missense mutations in our study showed a 22±9% reduction in cMyBP-C protein level compared with donors.

Correction of ADP-sensitivity in HCM with mutant cTnT by human recombinant wild-type troponin

To evaluate whether mutant thin-filament protein directly causes destabilization of the B-state and changes sensitivity to ADP, troponin exchange experiments were performed in cardiomyocytes from the *TNNT2*$_{mut}$ sample. Because the homozygous *TNNT2*$_{mut}$ results in 100% mutant cTnT protein, it provides the unique opportunity to control the amount of wild-type human troponin complex incorporated.

Exchange was performed with 1 mg/mL of wild-type human troponin complex and resulted in 85±2% troponin exchange based on Western blot analyses of endogenous and myc-tag labeled wild-type cTnT (Fig. 5A). We have previously shown that this protocol does not affect other sarcomeric (phospho)proteins.[8,9] In exchanged cells without PKA treatment, replacement of mutant troponin with

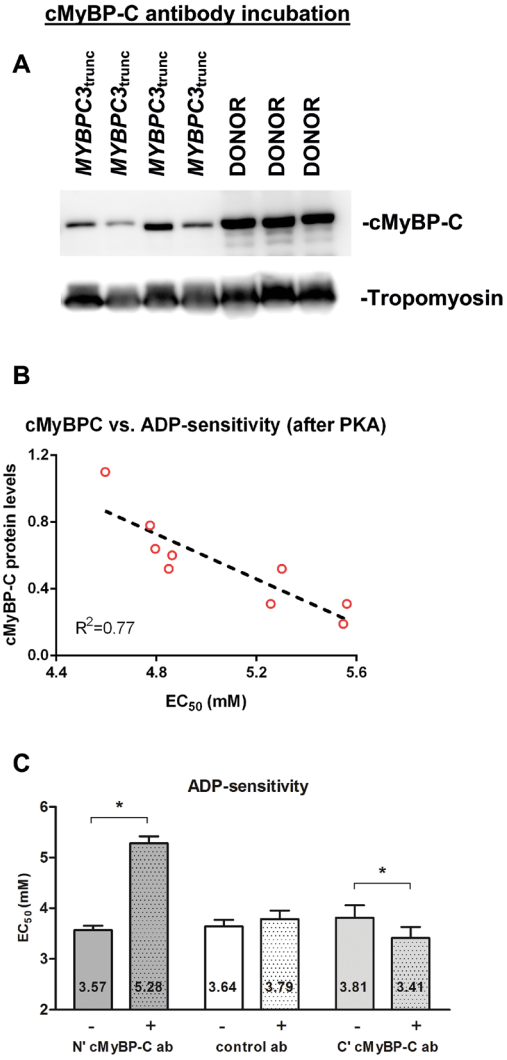

Figure 4. Cardiac myosin-binding protein-C (cMyBP-C) regulates ADP-sensitivity. A) cMyBP-C protein levels were assessed in *MYBPC3*$_{trunc}$ (n=9) and Donor (n=3) samples. Western blots were stained with specific antibodies for total cMyBP-C and tropomyosin (loading control). No traces of truncated cMyBP-C proteins were found in *MYBPC3*$_{trunc}$ samples. (B) Expression level of cMyBP-C was significantly lower in *MYBPC3*$_{trunc}$ than in donors. cMyBP-C protein levels from *MYBPC3*$_{trunc}$ samples were plotted against their corresponding sample average for ADP-sensitivity (after protein kinase A (PKA) treatment). (C) Single membrane-permeabilized cardiomyocytes isolated from idiopathic dilated cardiomyopathy heart samples were incubated with a specific cMyBP-C N'-terminal (mouse monoclonal) antibody, cMyBP-C C'-terminal (rabbit polyclonal) or a control (mouse monoclonal) antibody against nucleophosmin (antibodies used at 1:100 dilutions). Myofilament ADP-sensitivity (EC_{50}) before (-) and after (+) treatment with cMyBP-C antibody (N' or C' cMyBP-C ab) or nucleophosmin antibody (control ab). Myofilament ADP-sensitivity was significantly reduced after N' cMyBP-C ab treatment, but not after C' cMyBP-C ab or control ab. Data were compared using Paired samples t-Test for each specific group of antibodies. 8-11 cardiomyocytes from 3 to 4 failing heart samples were used per group.

Table 3. Response of ADP-stimulated contraction with cardiac myosin-binding protein C (cMyBPC) and control antibodies.

Sample	Before	After	ns
	N′ cMyBPC ab (2.2 µm)	N′ cMyBPC ab (2.2 µm)	
IDCM			N=4, n=11
ADP-F_{act} (kN/m^2)	8.4±1.0	6.6 ±0.9*	
ADP-F_{pass} (kN/m^2)	0.36±0.1	0.33±0.1	
	nucleolar ab (2.2 µm)	nucleolar ab (2.2 µm)	N=3, n=8
IDCM			
ADP-F_{act} (kN/m^2)	9.3±1.1	9.0±1.0	
ADP-F_{pass} (kN/m^2)	0.57±0.1	0.50±0.1	
	C′ cMyBPC ab (2.2 µm)	C′ cMyBPC ab (2.2 µm)	N=3, n=11
IDCM			
ADP-F_{act} (kN/m^2)	11.7±1.9	10.8±2.0	
ADP-F_{pass} (kN/m^2)	1.5±0.3	1.2±0.2*	

Paired samples t-Test. $p<0.05$ was considered significant; *vs before antibodies; ab, antibody; C′ cMyBPC or nucleolar antibody served as controls for unspecific binding; N= number of samples; n= number of cardiomyocytes; 2.2 µm sarcomere length; IDCM, idiopathic dilated cardiomyopathy; ADP-F_{act}, ADP total tension (10 mM ADP); ADP-F_{pass}, ADP passive tension

recombinant unphosphorylated wild-type troponin did not restore the ADP-sensitivity (Fig. 5B, 3.16±0.16 mM ADP) to donor levels (Fig. 3B, 3.96±0.15 mM ADP). Notably, PKA treatment in exchanged cells containing 85% wild-type troponin complex restored ADP-sensitivity (Fig. 5B, 4.16±0.24 mM ADP) to donor levels that were PKA-treated (Fig. 3C, 4.38±0.16 mM ADP). Control *TNNT2*$_{mut}$ cardiomyocytes, in the presence of exchange buffer without wild-type troponin complex showed similar ADP-sensitivity (Fig. 5B, 2.86±0.07 mM ADP before PKA and 2.98±0.05 mM ADP after PKA) as *TNNT2*$_{mut}$ cardiomyocytes without the exchange procedure (Fig. 3B and 3C, 3.00±0.17 mM ADP before PKA and 3.34±0.13 mM ADP after PKA). Together, these results support that this specific cTnT mutant protein disrupts tropomyosin's B-state position on actin and hence, is highly susceptible for ADP-activated myosin-binding.

Physiological ADP levels increase Ca^{2+}-sensitivity in idiopathic dilated and hypertrophic cardiomyopathy

We recently provided evidence that elevation of myocardial ADP levels, even in the µmolar range, in the presence of diastolic Ca^{2+}, contribute to diastolic dysfunction by increasing residual actin-myosin interactions and elevating myofilament Ca^{2+}-sensitivity.[17] This leads to high cardiomyocyte stiffness and abnormal relaxation, associated with limited ventricular compliance.[17] In HCM animal models an increased

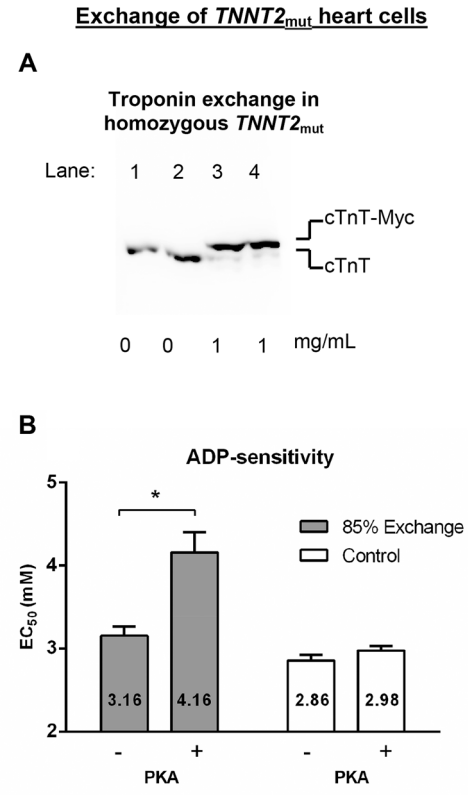

Figure 5. Cardiac troponin T (cTnT) regulates ADP-sensitivity. Endogenous mutant cTnT in *TNNT2*$_{mut}$ cardiomyocytes was exchanged by exogenous recombinant human wild-type troponin complex. *TNNT2*$_{mut}$ cells were incubated with 1 mg/mL wild-type human troponin complex or without troponin complex (Control). (A) Quantification of troponin exchange in cardiomyocytes from a *TNNT2*$_{mut}$ heart. Immunoblots stained with a specific antibody against cTnT that recognizes both endogenous mutant cTnT (lower band) and recombinant myc-tag wild-type cTnT (cTnT-myc; upper band). *TNNT2*$_{mut}$ without recombinant troponin complex (lanes 1 and 2; Control cells) and with 1 mg/mL recombinant wild-type troponin complex (lanes 3 and 4). Exchange resulted in 85±2% troponin exchange based on Western blot analyses of endogenous and myc-tag labeled wild-type cTnT. (B) Myofilament ADP-sensitivity (EC_{50}) before (-) and after (+) protein kinase A (PKA)-treatment. ADP-sensitivity was significantly reduced after PKA treatment in troponin-exchanged cardiomyocytes. No change in myofilament ADP-sensitivity was observed in control cardiomyocytes from the *TNNT2*$_{mut}$ heart. Data were compared using 2-way ANOVA followed by Bonferroni *post-hoc* test. 4 cardiomyocytes from 1 *TNNT2*$_{mut}$ heart were used per group.

[ADP] was observed from ~60 μM in controls to ~100 μM in diseased hearts.[28-30] We therefore investigated if an ADP level as reported in diseased hearts (100 μM) in concert with Ca^{2+} increases force generation in human membrane-permeabilized cardiomyocytes from IDCM and HCM hearts compared to non-failing donor myocardium. Figures 6*A* and 6*B* show normalized force-Ca^{2+} relations in the presence of 100 μM ADP, before and after PKA treatment, for *MYH7*$_{mut}$ and *MYBPC3*$_{mut}$ samples.

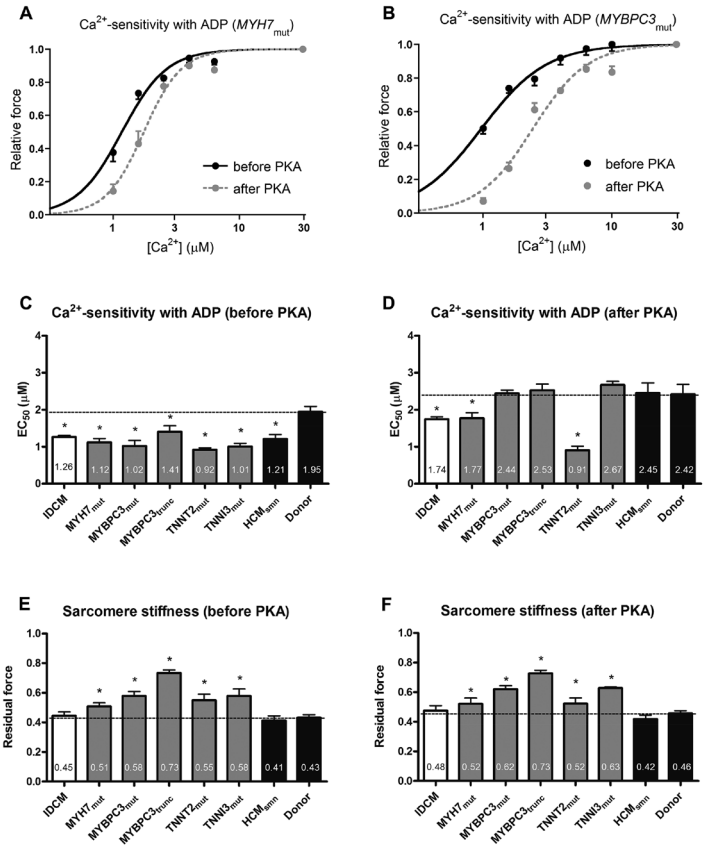

Figure 6. Myofilament Ca^{2+}-sensitivity and sarcomere stiffness, in the presence of 100 µM ADP, in idiopathic dilated (IDCM) and hypertrophic (HCM) cardiomyopathy membrane-permeabilized cardiomyocytes. Normalized force-Ca^{2+} relationships before and after protein kinase A (PKA) in the presence of ADP for *MYH7*$_{mut}$ (*A*) and *MYBPC3*$_{mut}$ (*B*) heart samples. Myofilament Ca^{2+}-sensitivity in the presence of ADP (EC_{50}) before (*C*) and after (*D*) PKA treatment in IDCM, HCM and donor samples. Residual force, a measure of sarcomere stiffness, at high Ca^{2+} with 100 µM ADP (EC_{50}) before (*E*) and after (*F*) PKA treatment in IDCM, HCM and donor samples. Data was compared using multilevel analysis. N= number of samples; n= number of cardiomyocytes. IDCM (N=4, n=18) *MYH7*$_{mut}$, myosin heavy chain mutations (N=3, n=10); *MYBPC3*$_{mut}$, myosin-binding protein-C (missense) mutations (N=3, n=10); *MYBPC3*$_{trunc}$, myosin-binding protein-C (truncating) mutations (N=4, n=12); *TNNT2*$_{mut}$, cardiac troponin T mutation (N=1, n=4); *TNNI3*$_{mut}$, cardiac troponin I mutation (N=1, n=4); HCM$_{smn}$, sarcomere mutation-negative HCM samples (N=3, n=8). 14 cardiomyocytes from 5 non-failing hearts (donor) served as controls.

In the presence of 100 µM ADP, myofilament Ca^{2+}-sensitivity was significantly higher in IDCM and HCM samples compared to donors (Fig. 6*C*). Because we have previously demonstrated that the high myofilament Ca^{2+}-sensitivity in human cardiomyopathy samples correlates well with the low phosphorylation of PKA myofilament targets[4,9], we repeated the measurements after treatment with exogenous PKA. PKA treatment

normalized the high Ca^{2+}-sensitivity in HCM with *MYBPC3* and $TNNI3_{mut}$ mutations and HCM_{smn}, while Ca^{2+}-sensitivity remained higher in IDCM, $MYH7_{mut}$ and $TNNT2_{mut}$ samples (Fig. 6*D*). In addition, we observed augmented sarcomere stiffness for all sarcomere-mutation positive HCM samples evidenced by high residual force compared to non-failing donors (Fig. 6*E*). Residual force values in IDCM and HCM_{smn} were similar to those observed in non-failing donor cells before and after PKA treatment (Figs. 6*E* and 6*F*). The increase in sarcomere stiffness suggests an increased strain for each individual cross-bridge and/or an enhanced number of actin-myosin interactions for HCM samples with sarcomeric mutant proteins.

DISCUSSION

In this study we show that ADP-stimulated contraction can be used as experimental tool to assess changes in thin-filament transitions induced by sarcomeric mutations and phosphorylation-mediated protein alterations. These mutation- and phosphorylation-related changes in ADP-sensitivity are explained by changes in tropomyosin's position and the number of accessible myosin-binding sites on actin. In accordance with our first hypothesis, we show that HCM cardiomyocytes containing troponin mutations have high ADP-sensitivity, irrespective of the phosphorylation background. High ADP-sensitivity was corrected to control values upon incorporation of healthy troponin in HCM cardiomyocytes harboring a homozygous $TNNT2_{mut}$ mutation. These data indicate that the troponin mutations investigated in the present study disrupt the steric blockade imposed by tropomyosin, which favors accessibility of myosin-binding sites on actin. The high responsiveness to ADP observed in IDCM, $MYH7_{mut}$ and HCM_{smn} is, in this context, solely explained by reduced PKA-phosphorylation of myofilament proteins. This is consistent with the idea that PKA phosphorylation of the myofilaments stabilizes the B-state thin-filament formation, which reduces myosin-binding sites on actin. Moreover, in agreement with our second hypothesis HCM samples with *MYBPC3* mutations were shown to have less sensitivity to ADP compared with cells from non-failing hearts, in support for the enhanced stabilization of the steric blockade of tropomyosin.

Finally, a pathophysiologic level of ADP elevated myofilament Ca^{2+}-sensitivity in all disease samples. Furthermore, under these conditions, high myofilament stiffness was observed in samples from HCM patients with sarcomeric mutations. The high myofilament Ca^{2+}-sensitivity in the presence of ADP was corrected to control values by PKA in all HCM samples, except for the samples with *MYH7* and *TNNT2* mutations, while myofilament stiffness was unaffected by PKA in all mutation-positive HCM samples. These data illustrate the complex interaction between changes in protein phosphorylation, calcium and ADP. Overall, our data support the notion that increased actin-myosin interactions during the diastolic phase may occur in environments where the myofilaments are highly sensitized by the complex interactions of low phosphorylation of PKA-targets, elevated *in vivo* levels of ADP and of diastolic Ca^{2+}, which may limit muscle relaxation.

Modulation of ADP-sensitivity by PKA-mediated phosphorylation

We showed that PKA-mediated phosphorylation of the myofilaments reduced the responsiveness to ADP, supporting the stabilization of the B-state formation (Figs. 2 and 3C). In the intact heart, regulation of $[Ca^{2+}]_i$ transients is accomplished via sympathetic stimulation of β-adrenergic receptors, which results in positive inotropic (contraction), lusitropic (relaxation) and chronotropic (frequency) effects.[31] At the myofilament level, PKA main targets are cTnI, cMyBP-C and titin. Multiple roles have been assigned to increased PKA-phosphorylation of myofilament proteins, such as reduced myofilament Ca^{2+}-sensitivity, increased length-dependent activation (cellular basis of the Frank-Starling relation) and enhanced cross-bridge cycling kinetics via phosphorylation of cTnI and cMyBP-C.[5-8]

The reduced ADP-sensitivity following PKA incubation implies strengthening of the B-state localization of tropomyosin. There are likely mechanisms, which may act synergistically, that could explain this effect:

Firstly, the reduction in ADP-sensitivity may be explained by cTnI phosphorylation. Two sites on cTnI are known to be target of PKA-induced phosphorylation in human, Serines 23 and 24.[32,33] Phosphorylation of these sites is associated with reduced Ca^{2+}-affinity of troponin C (cTnC)[34] due to structural changes of cTnC that diminish its Ca^{2+}-affinity[35-37]. Although cTnC changes are likely sufficient to account for the reduction of myofilament Ca^{2+}-sensitivity upon PKA activation, they do not necessarily apply to the present conditions where Ca^{2+} is absent. It has been shown that the C-terminal half of cTnI docks the troponin-tropomyosin complex onto the outer domain of actin at low cytoplasmic $[Ca^{2+}]$[38], thereby stabilizing the formation of the B-state. The reduced ADP-sensitivity after PKA treatment likely reflects potentiation of the inhibitory function of cTnI-tropomyosin on actin as PKA decreases the affinity of cTnI for cTnC. The PKA-mediated conformational changes would increase tropomyosin-mediated blockade of myosin-binding sites on actin.

Secondly, PKA-mediated phosphorylation of cMyBP-C may underlie the observed reduction in ADP-sensitivity. PKA-phosphorylation of cMyBP-C occurs at the M-domain where it interacts with actin.[39] It was shown that M-domain phosphorylation reduces the N-terminal affinity of cMyBP-C for actin[40], consistent with less activation of the thin-filament[41]. One can speculate that cMyBP-C phosphorylation reduces competition between cMyBP-C and tropomyosin on actin, and subsequently decreases the number of myosin-binding sites on actin.

Modulation of ADP-sensitivity by the N' and C' terminus of cMyBP-C

In the heart, up to 9 transverse stripes (half-sarcomere) of cMyBP-C are observed in the C-zone, where it is recently been proposed to modulate myofilament activation.[42] The C-terminus of cMyBP-C is important for proper localization and anchoring onto the thick-filament and the rod region of myosin.[43,44] The N-terminal region has been shown to directly interact with actin in *in vitro* studies[45,46] and *in situ* 3D reconstructions, where it was shown to regulate thin-filament transitions via direct competition for

the low Ca^{2+}-state (B-state) position with tropomyosin[20,21]. Here we provide further evidence for N-terminal cMyBP-C's interaction with tropomyosin by showing that cMyBP-C regulates thin-filament transitions via its N-terminal region (Fig. 4C). Pre-incubation of IDCM myofilaments with a N-terminal cMyBP-C antibody is expected to decrease cMyBP-C's affinity to bind and compete with tropomyosin and shift it towards the C-state. Indeed, N-terminal cMyBP-C antibody pre-incubation decreased ADP-sensitivity in membrane-permeabilized cardiomyocytes, consistent with less myosin-ADP binding to actin. In addition to its effect on the thin-filament, there is general agreement that cMyBP-C acts as brake on myosin cross-bridge cycling.[47-49] This effect is mediated by the interaction of cMyBP-C's N' terminus (C1-C2) with subfragment 2 (S2) of myosin[50], whereby cMyBP-C is able to slow cross-bridge cycling kinetics. Interestingly, incubation of membrane-permeabilized cardiomyocytes with specific antibody binding to the C' terminus of cMyBP-C increased the responsiveness to ADP (Fig. 4C). This might be due to allosteric protein changes that translate their effects from the C-terminal domain of cMyBP-C to its N-terminal region, which release part of the brake on myosin.

Haploinsufficiency of cMyBP-C reduces ADP-sensitivity of myofilaments

Our data indicate that loss of cMyBP-C full length protein (i.e. haploinsufficiency) in HCM with truncating cMyBP-C mutations weakens cMyBP-C-tropomyosin interactions and result in a reduced accessibility of myosin-binding sites on actin, evident from a reduced ADP-sensitivity (Figs. 3B and 3C). This is consistent with the recent observation that the N-terminal region of cMyBP-C binds to the low Ca^{2+}-state position of tropomyosin (B-state), which is sufficient to interfere and compete with tropomyosin-actin interactions, moving tropomyosin into the C-state position.[20,21] A schematic picture is presented in Figure 7A to illustrate that the loss of cMyBP-C full length protein weakens the overall cMyBP-C-tropomyosin interaction resulting in reduced accessibility of myosin-binding sites on the actin filament in the absence of Ca^{2+}. In contrast, the presence of cMyBP-C competes with tropomyosin for the B-state position on actin and increases the accessibility of myosin-binding sites on the actin filament (Fig. 7B). Reduced ADP-sensitivity was also observed in cMyBP-C HCM samples with missense mutations, where loss of full length protein may similarly decrease the availability of myosin-binding sites on actin. However, in these samples cMyBP-C poison peptide effects on muscle function cannot be excluded. The mutant protein could directly interfere with cMyBP-C-tropomyosin interactions. One can speculate that in particular the E258K mutation (localized on the actin-binding site[51]) has the potential to reduce its actin-affinity and thereby alter cMyBP-C-tropomyosin competition.

Increased diastolic actin-myosin interactions in cMyBP-C haploinsufficient tissue

While ADP responsiveness of myofilaments from HCM with *MYBPC3* mutations is reduced (Fig. 2B), myofilament sensitivity to Ca^{2+} is increased in the presence of pathophysiological levels of ADP (Fig. 5C). In the absence of Ca^{2+} and with low levels of cMyBP-C, an increased pool of myosins is less restrained by cMyBP-C at the thick-filament C-zone area. However, the myosin-binding sites on actin are less accessible due to the higher steric blockade as a result of reduced cMyBP-C-tropomyosin interactions (Fig. 6*A*). Hence, fewer actin-myosin interactions are formed. In the presence of Ca^{2+}, Ca^{2+} binding to troponin causes the movement of tropomyosin on actin and leads to the uncovering of myosin-binding sites. The reduced restraining effect on myosin caused by the low levels of cMyBP-C in combination with increased accessibility of myosin-binding sites on actin cause an increased myofilament Ca^{2+}-sensitization. Based on our findings this suggests that even low levels of Ca^{2+}, as those that exist *in vivo* during the diastolic phase, are sufficient to increase actin-myosin interactions in HCM heart with *MYBPC3* mutations and may be capable of limiting relaxation during diastole. This is indeed observed in cMyBP-C KO mice with a HCM phenotype, where cross-bridge cycling is accelerated in membrane-permeabilized cardiomyocytes[47,48], consistent with impaired relaxation of intact cardiomyocytes[52]. Pohlmann et al.[52] observed that cardiomyocytes with total cMyBP-C ablation contracted at very low Ca^{2+} levels and had lower diastolic sarcomere length, that was associated with high actin-myosin interactions (i.e. diastolic length was partially normalized to controls following addition of the cross-bridge inhibitor BDM). This is also consistent with the augmented sarcomere stiffness observed in *MYBPC3* mutations (Fig. 5E) that could not be rescued after PKA treatment (Fig. 5F). Although counter-intuitive with the findings in the absence of Ca^{2+} (Fig. 2), where cross-bridge formation is reduced, it likely reflects the complex interaction of the factors discussed above, including thin-filament accessibility and Ca^{2+}-responsiveness. Overall, the results supports the concept that cMyBP-C restrains actin-myosin interactions at low Ca^{2+} to allow complete relaxation and filling during diastole.

Troponin mutants increase ADP-sensitivity of myofilaments

Here we provide evidence that mutations in troponin subunits may increase the availability of myosin-binding sites on actin, illustrated by higher myofilament ADP-sensitivity (Fig. 3B). A schematic picture is presented in Figure 7C to illustrate that more myosin-binding sites are accessible due to the greater disruption of the B-state caused by troponin mutations. Notably, troponin exchange experiments provide the direct proof that the endogenous mutant cTnT, used in this study, impairs tropomyosin's B-state position (Fig. 5B). This is in good agreement with solution studies with reconstituted HCM mutant troponin-tropomyosin proteins (in cTnI[19] and cTnT[18]) that support for a disrupted B-state. The C-terminal half of cTnI (residues 137-210) docks the troponin-tropomyosin complex onto the outer domain of actin at low [Ca^{2+}] and thereby maintains formation of the B-state.[38] It is likely that the present

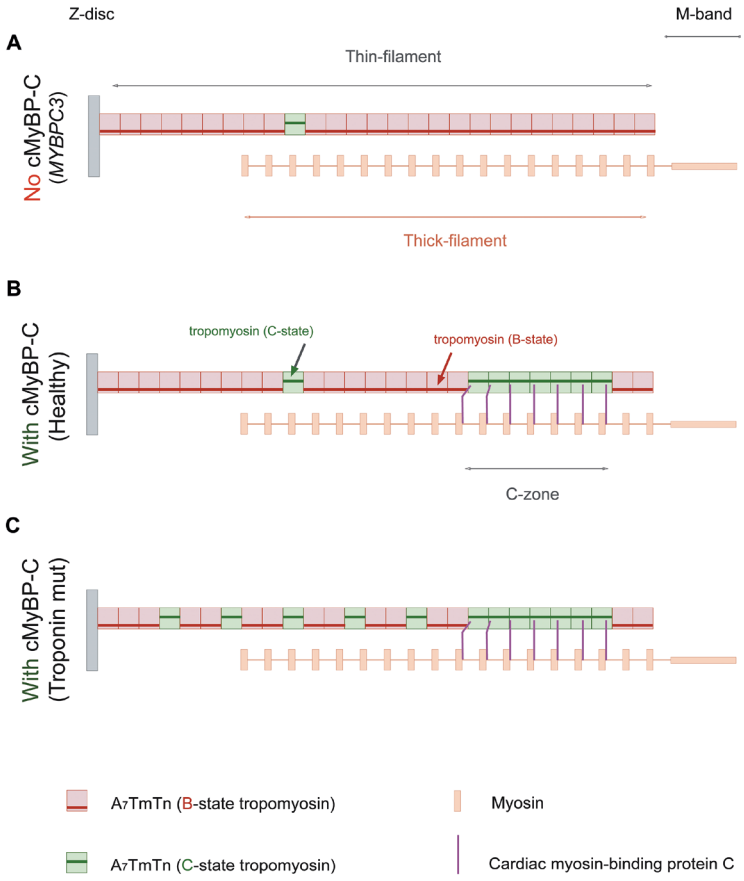

Figure 7. Schematic diagram of a half-sarcomere illustrating thin-filament transitions, in the absence of Ca^{2+}. The average length of the thin-filament (half-sarcomere) is 1.05 μm[53,54], which consists of a 38.5 nm periodicity of the actin-tropomyosin-troponin (A_7TmTn, functional unit) complex[55,56], i.e. each half thin-filament consists of 27 A_7TmTn units (red and green rectangular boxes depict functional units). The length of the thick-filament is ~1.63 μm. After subtracting the thick-filament bare zone (M-band region that is deprived of myosin heads; 0.16 μm), the thick-filament length per half-sarcomre is ~0.74 μm[57]. Since the distance between myosin helical repeats is 42.9 nm (on the ame axis layer)[58], ~18 myosins occupy each half-thick filament. (*A*) Solution studies indicate that ~95% of the myofilaments in the free Ca^{2+} state are blocked (B-state) in the absence of cardiac myosin-binding protein-C (cMyBP-C)[10]. This indicates that ~26 A_7TmTn units are blocked (depicted in red) and only a single unit of A_7TmTn is in the C-state position (depicted in green) per half-thin filament. The single "C"-state functional unit is randomly drawn in the scheme. This situation parallels cMyBP-C truncating mutations, where reduced total cMyBP-C protein levels have been found.[22,23] Please note that for clarity no cMyBP-C is shown (i.e. mimicking full lack of cMyBP-C). (*B*) In the presence of cMyBP-C more myosin-binding sites are available on actin, since the N-terminal extension of cMyBP-C dislocates tropomyosin into the C-state position[20,21] (depicted as 7 green functional units). Assuming that cardiac muscle contains a minimum of 7 cMyBP-C at regular intervals of 43 nm starting from "stripe" 5 to 11 in the C-zone (215 nm from the M-band)[59], cMyBP-C would coincide with the 2nd to 3rd myosin counting from the M-band in the scheme. This situation mimics healthy donor samples. (*C*) Mutations in troponin subunits disrupt troponin-tropomyosin interactions, which causes the thin-filament to be less blocked. As shown in the picture more functional units are in the C-state.

cTnI mutation (R145W) disrupts cTnI-tropomyosin interactions, which increases the availability of myosin-binding sites on actin. Likewise, cTnT via its interaction with tropomyosin stabilizes the thin-filament in the B-state[60,61], supporting that, at least the current cTnT mutation may confer a greater availability of myosin-binding sites on actin. Altogether these findings indicate that mutations in troponin proteins may disrupt the blockade of tropomyosin on actin, which precede the rise of cytosolic Ca^{2+}, and are able to augment actin-myosin interactions during the diastolic phase in HCM patients.

Clinical implications

Our study shows that the high basal sarcomeric activation previously observed in animals and humans with HCM[9,62-64] with thin-filament mutations may be partly due to the diminished steric blockade of myosin-binding sites on actin by tropomyosin that precede the rise in cytosolic Ca^{2+} (Fig. 3B). Our data illustrate that this effect is exacerbated by the diminished PKA protein phosphorylation in HCM and IDCM hearts (Fig. 3C). The high myofilament Ca^{2+}-sensitivity in patient samples with low PKA phosphorylation in the presence of pathologic ADP levels (Fig. 6) may be sufficient to slow ventricular relaxation. Additionally, sarcomere mutation-positive HCM samples show augmented sarcomere stiffness, which is not caused by low PKA phosphorylation (Figs. 6E and 6F). The increased myofilament Ca^{2+}-sensitivity and high sarcomere stiffness in the presence of ADP is in line with our previous study, where we showed that the complex interactions of Ca^{2+} and ADP increased myofilament Ca^{2+}-sensitivity and stiffness in membrane-permeabilized rat cardiomyocytes, limited restoration of diastolic length in intact rat cardiomyocytes and diminished ventricular compliance in whole rat hearts.[17] Altogether these myofilament changes have the potential to delay the onset of ventricular relaxation and limit proper filling, which are seen in patients[65,66] and animal models[62,63] of HCM, and human IDCM[4].

CONCLUSION

We conclude that a rather straightforward activation assay with ADP, in a membrane-permeabilized muscle preparation, can be used to reveal conformational changes in the 3-state model of thin-filament activation induced by mutations and post-translational modifications such as phosphorylation. The ADP-stimulated contraction assay indicates that increased force at diastolic Ca^{2+} levels, as observed in cardiomyopathies, involves increased cross-bridge formation, and is not restricted to changes in Ca^{2+}-binding affinity of cTnC. In addition, we show that pathologic ADP levels increase myofilament Ca^{2+}-sensitivity and stiffness, which may together contribute to the diastolic dysfunction seen in acquired and genetics forms of cardiomyopathy in humans.

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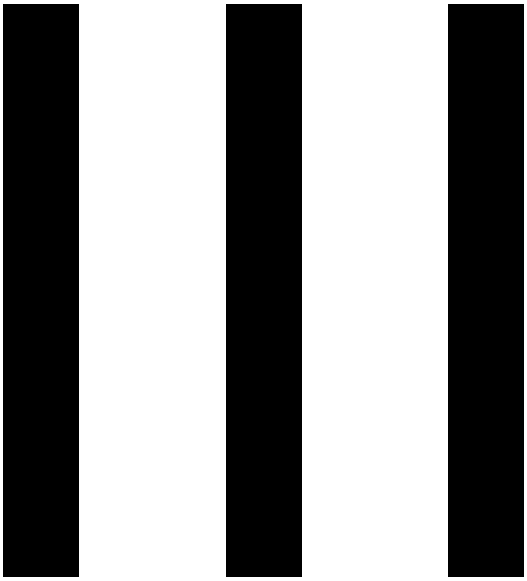
Author Contributions

All authors approved the final version of the manuscript. Conception and design of the experiments: V.S. devised, designed and performed the project, analyzed data and wrote the manuscript; A.N. performed experiments, analyzed data and revised the manuscript; P.J.M.W. performed experiments, analyzed data and revised the manuscript; C.d.R. collected the human cardiac samples and revised the manuscript; M.M. collected the human cardiac samples and revised the manuscript; D.W.D.K. supervised the project, wrote and revised the manuscript; J.v.d.V. supervised and funded the project, wrote and revised the manuscript.

Conflict of Interest

None to disclose.



SUMMARY, CONCLUSIONS *& CURRICULUM VITAE*

7

SUMMARY, CONCLUSIONS & FUTURE PERSPECTIVES

"All truths are easy to understand once they are discovered; the point is to discover them."

Galileo Galilei

SUMMARY

The purpose of this thesis was to dissect the cellular and mechanical alterations related to myocardial inactivation that limit diastolic performance in patients with familial hypertrophic cardiomyopathy (HCM) and patients with non-familial causes of heart failure. From experiments at the cellular and whole organ level, in humans and rats, we are able to translate our findings into the *in vivo* human setting to obtain a better understanding of human cardiac performance.

High myofilament Ca^{2+}-sensitivity is a common characteristic in human HCM

Significant efforts have been made to identify a common pathomechanism triggered by mutations that could explain the multitude of intracellular events observed in HCM. Impaired relaxation is a general finding in humans and animals carrying HCM gene mutations. Impaired diastolic function is observed before the development of hypertrophy and fibrosis. Impaired relaxation may be explained by the elevated myofilament Ca^{2+}-sensitivity (the "high Ca^{2+}-state"), which is observed in the majority of *in vitro* and transgenic mice studies. The high myofilament Ca^{2+}-sensitivity would be sufficient to cause 'systolic' activation at low [Ca^{2+}] capable of delaying the onset of relaxation. In Chapter 3 we performed a comprehensive study on a large set of human HCM samples to identify if high myofilament Ca^{2+}-sensitivity is characteristic of human HCM with mutations in thick- and thin-filament proteins. Since high myofilament Ca^{2+} sensitization depends on muscle length and the phosphorylation state of myofilaments, cardiomyocyte force measurements were performed at two different sarcomere lengths (i.e. length-dependent activation), without and with treatment with exogenous protein kinase A (PKA). Measurements were performed in HCM samples and compared with sarcomere mutation-negative and non-failing donors. We observed that high myofilament Ca^{2+}-sensitivity is a common characteristic of human HCM, though partly reflects the hypophosphorylated state of PKA targets and cannot solely be related to the mutant protein itself. In addition, reduced length-dependent myofilament Ca^{2+}-activation was shown to represent a common pathomechanism in HCM with missense mutations, which is independent of the phosphorylation background. Moreover, a blunted increase in maximal force development upon an increase in sarcomere length was found in HCM carrying missense mutations. The diminished length-dependent myofilament Ca^{2+}-activation was not rescued upon PKA administration in HCM with sarcomeric mutations. Finally, we provided direct proof that mutant troponin T (at least for the K280N) impairs length-dependent activation, supporting that mutations affect thin-filament geometry.

High myofilament Ca^{2+}-sensitivity and length-dependent activation are regulated by PKA and PKC phosphorylation of cardiac troponin I

In Chapter 4 the effects of troponin I (cTnI) phosphorylation at threonine 143 (Thr143) site by protein kinase C (PKC) on myofilament Ca^{2+}-sensitivity and length-dependent activation were studied. Because Thr143 is a well characterized PKC-phosphorylation site that is highly phosphorylated in human failing hearts, we investigated if phosphorylation of Thr143 modifies myofilament Ca^{2+}-sensitivity and length-dependent activation in human cardiomyocytes. PKC-mediated phosphorylation at Thr143 may be detrimental for the diastolic phase. The effects of Thr143 phosphorylation were compared with the well-known protein kinase A (PKA) phosphosites serines 23 and 24 (Ser23/24) of cTnI. Troponin exchange experiments were performed in membrane-permeabilized human cardiomyocytes. Isometric force was measured at various [Ca^{2+}] in exchanged cardiomyocytes with recombinant wild-type (Wt) troponin or troponin mutated at the PKC site Thr143, or Ser23/24 into aspartic acid (D) or alanine (A) to mimic phosphorylation and dephosphorylation, respectively. In troponin-exchanged donor cardiomyocytes experiments were repeated after incubation with exogenous PKA. Pseudo-phosphorylation of Thr143 site increased Ca^{2+}-sensitivity compared to controls (Wt) without affecting length-dependent activation of control cardiomyocytes. Subsequent PKA treatment enhanced the length-dependent shift in Ca^{2+}-sensitivity after Wt and 143D exchange. Exchange with Ser23/24 phosphosites demonstrated that pseudo-phosphorylation of both Ser23 and Ser24 sites is required for the length-dependent increase in Ca^{2+}-sensitivity. cTnI pseudo-phosphorylation did not alter length-dependent changes in maximal force. In conclusion, phosphorylation at Thr143 enhances myofilament Ca^{2+}-sensitivity without affecting length-dependent activation, while Ser23/24 bisphosphorylation is needed to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity. This study provides evidence that low PKA-mediated phosphorylation as observed in HCM cardiomyopathy may cause the high Ca^{2+}-sensitivity (the "high Ca^{2+}-state") and impair length-dependent activation

Energy depletion and diastolic dysfunction - detrimental effects of high ADP

On the basis of the previous findings that human HCM tissue confers a high degree of thin-filament activation, even at low Ca^{2+}, we sought to characterize the impact of sustained basal systolic stress in relation to myocardial energy deficiency. Increased myofilament ATP consumption has been observed in HCM animal models and our group recently confirmed this in human HCM patients, evident from the increased cost (ATP utilization) for myofilament contraction, i.e. reduced tension cost. The impaired tension cost may result from an increased Ca^{2+}-induced myosin-ATPase activity, which would decrease myocardial energy supply for other ATP-dependent processes in the cardiac muscle cells. There is a general consensus that the myocardial energy reservoir in HCM patients is reduced, as a result of reductions in ATP content, also termed the "myocardial energy depletion hypothesis". Although attractive, this hypothesis fails to address the paradigm that *in vivo* ATP levels at rest in animal models

of heart failure are mostly unchanged and that even during increased workloads, the [ATP] is never rate-limiting to power actomyosin interactions. To this end, in Chapter 5 we performed a basal study ranging from experiments at the cellular level to the whole organ, in humans and rats, as to provide the proof-of-concept that myocardial accumulation of ADP (instead of reduced ATP), even in the micromolar amount, can contribute to diastolic dysfunction. We provide evidence that synergistic actions of physiological levels of ADP and diastolic Ca^{2+} increase actomyosin interactions, which elevate myocardial stiffness and limit proper filling of the heart. Our findings show that increased cross-bridge interactions may lead to diastolic dysfunction in environments with elevated ADP and diastolic Ca^{2+}, evidenced by high cardiomyocyte stiffness and impaired diastolic re-lengthening, associated with limited ventricular compliance. In addition, we also showed that the contribution of cross-bridge interaction to total diastolic stress in rat membrane-permeabilized cardiomyocytes is much higher than previously considered. Perhaps most importantly, we showed that physiological levels of ADP (which increase strong-binding cross-bridge formation) are sufficient to increase myofilament Ca^{2+}-sensitivity and result in diastolic Ca^{2+}-overload. We speculate that the latter rise of diastolic Ca^{2+} can either result from the high Ca^{2+}-sensitivity that buffers more Ca^{2+} at the myofilaments and/or alterations in the ADP/ATP ratio that reduce the free energy released from ATP hydrolysis (ΔG_{ATP}) of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). Our study supports the idea that elevations of intracellular ADP, in specific types of cardiac disease where myocardial energy reserve is limited, contribute to diastolic dysfunction by recruiting cross-bridges even at low Ca^{2+} ("high Ca^{2+}-state").

High cross-bridge component in HCM

The fact that impaired relaxation is an almost universal finding in heart failure patients, and that this may result from inappropriate formation of force-producing cross-bridges during diastole ("high Ca^{2+}-state") lead us to study the mechanical changes associated with the blockade of actomyosin interactions, i.e. alterations in tropomyosin's position in Chapter 6. To this end, we investigated if the accessibility of myosin-binding sites on actin is altered in human idiopathic dilated cardiomyopathy (IDCM) and HCM samples. By measuring cardiomyocyte force-production in ADP-containing solutions (without Ca^{2+}) without and with exogenous PKA, the ability of myosin-ADP to bind non-blocked sites on actin was assessed. It is important to stress that any alteration in actomyosin interaction at this stage (no Ca^{2+}, but with ADP), precedes changes that occur in the presence of Ca^{2+} as occurs during muscle activation. In other words, an increased contribution of force-producing cross-bridges at the blocked state (B-state) will enhance the number of cross-bridges that are recruited in the presence of Ca^{2+}. Our study supports that diseased muscle has disrupted steric blockade of the thin-filament. This is either caused by the presence of mutant proteins, lack of protein in the case of cMyBP-C haploinsufficiency and/or reduced PKA-phosphorylation of myofilament proteins. Our mechanistic study supports the novel idea that the OFF

to ON transition of the thin-filaments is regulated by PKA-target phosphorylation and cMyBP-C. In addition, we show that, in the absence of Ca^{2+}, troponin mutations increase the actomyosin interactions; in contrast cMyBP-C and PKA both reduce accessibility of myosin-binding sites on actin.

Finally, cardiomyocyte from IDCM and HCM were shown to be more sensitive to Ca^{2+} in the presence of a pathologic level (100 μM) of ADP compared to controls. Exogenous treatment with PKA revealed that, except for troponin T mutations and myosin heavy chain mutations, all HCM samples were normalized to controls. Although PKA reduced myofilament Ca^{2+}-sensitivity in all samples, EC_{50} values in HCM in the presence of ADP (mimicking energy depletion) do not increase to the value observed in non-failing donor myocardium in the absence of ADP (i.e. healthy condition; Figure 1). In other words, our data suggest that even in environments where PKA-phosphorylation is preserved or normalized to controls, Ca^{2+}-sensitivity remains high due to an elevation of ADP. The detrimental effect of ADP in myofilament function is exacerbated when PKA-phosphorylation background is reduced compared to controls. In summary, our data support that enhanced actomyosin interaction contributes to diastolic dysfunction as myofilaments are highly sensitized by the synergistic actions of low phosphorylation of PKA-targets, elevated *in vivo* levels of ADP and of Ca^{2+}.

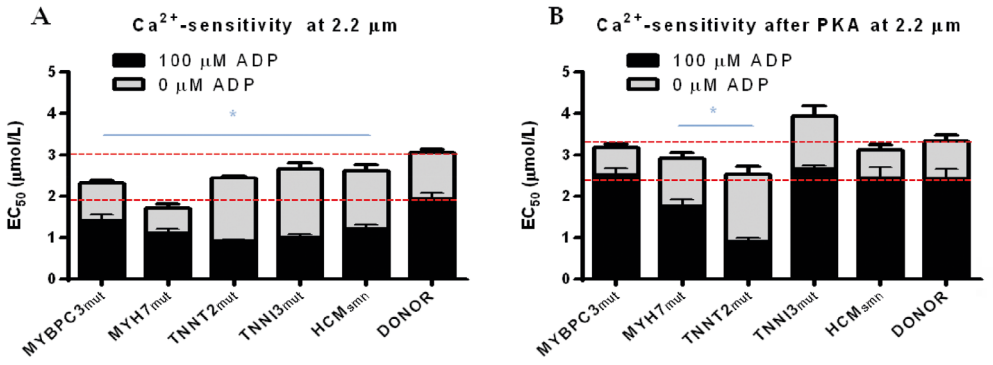


Figure 1. Myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm. Myofilament Ca^{2+}-sensitivity in the absence (gray bars) and presence of 100 μM ADP (black bars) in hypertrophic cardiomyopathy (HCM) cardiomyocytes. Myofilament Ca^{2+}-sensitivity before (A) and after (B) protein kinase A (PKA). Data was compared using multilevel analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; *TNNT2*$_{mut}$, cardiac troponin T mutation; *TNNI3*$_{mut}$, cardiac troponin I mutation; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Data from Chapter 3 and chapter 6 were combined to assess the effects of calcium in the absence (Chapter 3) and presence (Chapter 6) of a pathologic ADP level. Moreover, these graphs illustrate the effect of hypophosphorylation, which is frequently observed in cardiomyopathy. If we assume that ADP in HCM is high and phosphorylation of myofilament proteins is low, EC_{50} in HCM myocardium would be ~1 μM, compared to ~3 μM in non-failing donor myocardium with high PKA-phosphorylation and no ADP.

CONCLUSIONS

The main message of my studies is that a high level of cross-bridge interaction, which increases myofilament Ca^{2+}-sensitivity, contributes to force development during the diastolic phase. This may impair ventricular relaxation and limit ventricular filling. An optimal therapeutic strategy would be to reduce the 'high Ca^{2+}-state' and in parallel correct low PKA-phosphorylation and the elevated myocardial ADP. As highlighted, the 'high Ca^{2+}-sensitive' state has the potential to slow myocardial relaxation, limit diastolic re-lengthening and heart cavity size. These would reduce the Frank-Starling reserve and may ultimately lead to sudden cardiac arrest. Overall our data suggests that a combination of adrenergic and metabolic treatment might be beneficial for diastolic heart failure patients.

FUTURE PERSPECTIVES

Based on our recent findings published in Chapters 3, 5 and 6, we propose that high myofilament Ca^{2+}-sensitivity ('the high Ca^{2+}-state') is a common characteristic in human HCM, which may associate with high ADP levels. It is currently unclear why HCM patients would suffer from high myocardial ADP, but this may result from depleted myocardial energy reserve. Currently scarce data is available to explain why and how the energetic reserve is reduced in HCM individuals. There are several candidates that deserve focused attention, including creatine kinase (CK) and mitochondrial activity. Unpublished data from our group shows that CK expression is reduced in sarcomere-mutation positive HCM patients (Figure 2A).

This coincides with a substantial reduction in CK-activity compared with non-failing controls (Figure 2B). The change in protein expression was independent of changes that precede CK protein transcription, since mRNA of CK levels were not significantly altered in the majority of HCM samples. The only exception was a reduction in mitochondrial CK (CK-mt) mRNA in thin-filament mutations (Figure 3B).

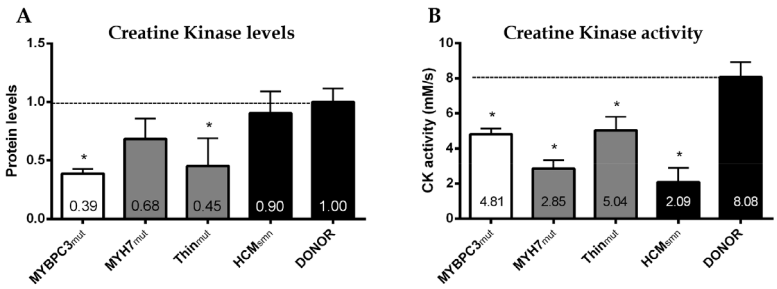


Figure 2. Creatine Kinase (CK) protein-expression (A) and activity (B) in human hypertrophic cardiomyopathy (HCM) samples. Data was compared using multilevel analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; Thin$_{mut}$, Thin-filament mutations consist of cardiac troponin T and cardiac troponin I mutations; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Unpublished data.

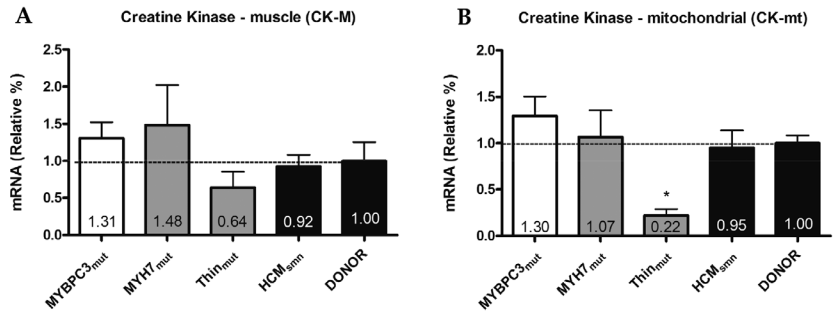


Figure 3. Creatine Kinase (CK) mRNA-expression of muscle (A) and mitochondrial forms (B) in human hypertrophic cardiomyopathy (HCM) samples. Data was compared using 1-way ANOVA analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; Thin$_{mut}$, Thin-filament mutations consist of cardiac troponin T and cardiac troponin I mutations; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Values were set to 1, relative to donor. Unpublished data.

In summary, the present data will strengthen the overall idea about the essential role of myocardial energetic reserve in HCM and will possibly provide alternative areas to carefully study in HCM pathophysiology. Measurements of mitochondrial activity require fresh human HCM samples. We will analyse mitochondrial oxygen consumption in our future studies. In addition, it is imperative to quantitatively measure the precise levels of ADP in the cell, which can already be accomplished nowadays with high-sensitive fluorescent biosensors, such as "PercevalHR". It is our belief that a complex of myofilament and Ca^{2+}-handling changes is triggered by deficient (mitochondrial) energetics in HCM. Future approaches should use animal models and test novel drugs to improve myocardial energetic reserve, including the promising SS-31 (Szeto-Schiller), a mitochondrial-targeted antioxidant peptide that promotes mitochondrial respiration and ADP regeneration to ATP. These have the future potential to partially rescue HCM progression in humans.

8

CURRICULUM VITAE

"Finally we shall place the Sun himself at the center of the Universe."

Nicolaus Copernicus

CURRICULUM VITAE

Vasco Sequeira Oliveira was born on January 14, 1986 in Porto, Portugal. In September 2004 he enrolled in a Bachelor degree in Biology at the Faculty of Sciences from the University of Porto – Portugal. During this time, he started working as a research intern at the Department of Physiology of the Faculty of Medicine of Porto under the supervision of Professor Adelino Leite-Moreira. Vasco finished his Bachelor in August, 2008 and enrolled in the same year in a Master's program in Molecular Genetics at the School of Sciences of the University of Minho – Portugal. He obtained his Master's degree title January, 2011 in a joint collaboration of the School of Sciences of the University of Minho and the Department of Physiology of the Faculty of Medicine of Porto. On February 24, 2011 Vasco arrived to Amsterdam and started as a PhD student at the Department of Physiology of the VU Medical Centre in Amsterdam under the supervision of Professor Jolanda van der Velden. As of June 1, 2016 he will be a postdoc fellow in the Department of Clinical Pharmacology of the Vanderbilt University Medical Center in Nashville, Tennessee – United States of America.

TRAINING

Cardiac function and Adaptation (PhD-training course of Dutch Heart Foundation). Papendal, Arnhem - Netherlands. October 2012

Scientific Poster Design (PhD-training course). ICaR-VU, Amsterdam - Netherlands. April 2012

Vascular Biology (PhD-training course of Dutch Heart Foundation). Papendal, Arnhem – Netherlands. October 2011

Course on Plasma Membrane Transporters (Post-graduated advanced training). School of Sciences, University of Minho, Braga – Portugal. July 2010

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Course on Scientific Illustration (Scientific Workshop). School of Sciences, University of Minho, Braga – Portugal. March 2008

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Teacher and supervisor 'Reduced length-dependent activation in human cardiomyocytes harbouring the cardiac Troponin I mutation R145W'' for international graduate student of Physiology. Department of Physiology, VU University Medical Center, Amsterdam – Netherlands. June-August 2011

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RESEARCH AWARDS

Best Poster Presentation Award. 33rd Meeting of the European Section of ISHR. University of Bordeaux, Bordeaux – France. July 2015

Best Poster Presentation Award. Modifiers and Modulators of Myofilament Function – awarded by Elsevier. Monona Terrace Convention Center, Madison – Wisconsin, United Sates. June 2014

European Society of Cardiology (ESC) First Contact Initiative Grant. Travel Grant awarded by the ESC Council on Basic Cardiovascular Science. August 2013

Young Investigator Prize (Poster presentation). Winter Meeting – Heart Failure Association (European Society of Cardiology). Les Diablerets – Switzerland. January 2012

VISITS ABROAD

Research guest at Professor Björn Knollmann's laboratory – Nashville – Tennessee, United States. September 2015 – January 2016

Visit to Bjorn Knollmann's group – Nashville – Tennessee, United States. February 2015

Visit to Jil Tardiff's group – Tucson – Arizona, United States. January 2014

ORAL COMMUNICATIONS

33rd Meeting of the European Section of ISHR. University of Bordeaux, Bordeaux – France. July 2015

Biophysical Society 59th Annual Meeting – Baltimore. Baltimore Conference Center, Baltimore – Maryland, United States. February 2015

43rd European Muscle Conference - Faculty of Natural Sciences, Salzburg – Austria. September 2014

Modifiers and Modulators of Myofilament Function – Madison. Monona Terrace Convention Center, Madison – Wisconsin, United States. June 2014

Biophysical Society 57th Annual Meeting – Philadelphia. Pennsylvania Conference Center, Philadelphia – Pennsylvania, United States. February 2013

3rd Rembrandt Symposium of the Rembrandt Institute for Cardiovascular Science. Leeuwenhorst Conference Center, Noorwijkerhout – Netherlands. November 2012

II Florence International Symposium on Advances on Cardiomyopathies – 9th Meeting of the European Myocardial and Pericardial Diseases WG of the ESC. Palazzo degli Affari, Florence – Italy. September 2012

Frontiers in CardioVascular Biology – 2nd Meeting of the ESC Council on Basic Cardiovascular Science. Imperial College, London - United Kingdom. April 2012

POSTER COMMUNICATIONS

33rd Meeting of the European Section of ISHR. University of Bordeaux, Bordeaux – France. July 2015

Modifiers and Modulators of Myofilament Function – Madison. Monona Terrace Convention Center, Madison – Wisconsin, United States. June 2014

The Academy Colloquium. Royal Netherlands Academy of arts and Science, Amsterdam – the Netherlands. March 2014

11th Dutch-German Joint Meeting. Molkenkur, Heidelberg – Germany. March 2013

VUmc Science Exchange Day. VU Medical Center, Amsterdam – Netherlands. March 2013

PhD-training course of Dutch Heart Foundation. Papendal, Arnhem – Netherlands. October 2012

Biophysical Society 56th Annual Meeting – San Diego. California Conference Center, San Diego – California, United States. February 2012

Winter Meeting – Heart Failure Association (European Society of Cardiology). Les Diablerets – Switzerland. January 2012

Rembrandt Symposium - Rembrandt Institute Symposium. Indisch Huis, Amsterdam – Netherlands. November 2011

PhD-training course of Dutch Heart Foundation. Papendal, Arnhem – Netherlands. October 2011

LIST OF PUBLICATIONS

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Now to my Robert! You, more than anyone, started to take care of me even before my flight first landed in the morning of the 24th February 2011.

From: vasco sequeira
Sent: 22.02.2011, 06:4:05 +0100
To: r.szulcek@vumc.nl
Subject: meeting
Dear Robert,
My flight arrives to Amsterdam at 2pm and the only place I know for sure where to go is the physiology department at the VU. Is it good for us to meet there?
thanks,
Vasco

From: r.szulcek@vumc.nl
To: vasco@vista.aero
Subject: RE: meeting
Date: Tue, 22 Feb 2011 07:39:21 +0100
Yes let us meet at the physiology. the secretary will tell you where to find me or you give me a call. safe trip. See you later.
Robert
PS: And don't forget warm cloths. It is freaking cold at the moment! :-(

There is nothing new I can say to you that I haven't said or expressed before. You are well aware of how important you have been to me since day -2, and how much we accomplished together (and still to come!). The lunches, the dinners, the vacations, the trips, the cycling, all the good and the bad moments we went through. You are another person I can call a brother of mine (yes, you need to adopt the cat as well ☺)

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Dank u wel/Thank you/Obrigado

Heart failure and diastolic dysfunction

The heart and a balloon have more in common than meets the eye. Like the heart, a balloon is a flexible bag that can be inflated and emptied. Using this example one can readily understand basic heart function and several reasons for failure. Consider a balloon that is filled with air for 10 seconds and then empties its volume for 10 seconds. The total time of balloon function or "balloon cycle" (filling and ejecting) is 20 seconds. Now let us imagine that the balloon cycle time of filling ("balloon filling") is reduced to 5 seconds; we have less time available to fill the balloon with air and hence the balloon will only be partially inflated. Thus a "balloon failure" condition appears. The ability to fill a balloon with air is also dependent on the properties of the material that it is made of. The thicker and more inflexible the material ("stiffer"[1]), the harder it will be to inflate ("balloon stiffness"), and a greater force or "stress"[2] must be applied to achieve the same results. This is analogous to a condition where the walls of the heart become thicker and/or increase in stiffness ("heart stiffness") and it gets harder for the heart to fill. Again the "failure" condition ensues. Importantly these processes can occur without changing the balloon filling time. Since both types of balloon failure described (balloon filling time and balloon stiffness) occur during the filling of the balloon we term it "balloon filling dysfunction".

The heart is not that different from a balloon, and a chronic perturbation of the heart cycle – "cardiac cycle" – is clinically classified as a cardiac disease or heart failure (HF) condition. Just in Europe over 15 million people are diagnosed with HF and this number continues to rapidly increase, representing a major cause of hospitalization and death. About half of the HF patient population have a heart which is unable to proper relax and distend (fill). Classically, heart function has been separated into several distinct phases. Even though the events of the cardiac cycle are mutually related, their *modus operandi* is distinct. Similar to the emptying and filling of the balloon, the heart needs to first stop "squeezing" (ejecting blood) so that it can start to relax – "myocardial relaxation" (inactivation). Only after the heart relaxes will it be able to fill and distend. During the filling phase alterations to the macro- (e.g. wall thickness, chamber geometry) or micro-properties (e.g. sarcomere stiffness and collagen deposition) of the tissue will change the heart's diastolic stiffness, stress and "elasticity"[3]. It takes a healthy heart ~0.1 seconds to completely relax (isovolumetric relaxation); and another ~0.4 seconds to fill and distend to a total of 120 ml of blood (filling phase) before "isometric contraction" takes place. Together these processes of relaxation and filling are known as the diastolic phase of heart function. Hence, perturbations or disruption to these processes lead to "DIASTOLIC DYSFUNCTION".

In this thesis, I have studied the mechanisms that affect myocardial relaxation and contribute to DIASTOLIC DYSFUNCTION of the heart. These include changes of "SARCOMERE LENGTH", "Ca^{2+}" and "MYOCARDIAL ENERGETICS".

A heart completely filled with blood is able to generate more pressure during contraction than a half full heart. This is because more of the heart muscle is engaged to contract and eject blood. This provides the basic frame work for what is known as the "FRANK-STARLING LAW". This Law relates the extent of heart filling (known as "length-dependency") with the amount of pressure generated during contraction. From these findings comes the universal principle that the amount of blood ejected from the heart must equal the amount of blood acquired during filling. The force for heart contraction is generated by the unitary building blocks of the heart muscle, which are known as "sarcomeres". Sarcomeres increase in length during filling which increases contractility of the heart cells. The term "SARCOMERE LENGTH-DEPENDENT ACTIVATION" describes the FRANK-STARLING LAW at the single cell level. Disruption of sarcomere lengthening function can result in improper filling of the heart (DIASTOLIC DYSFUNCTION) which affects the hearts ability to generate force and limits the amount of blood pumped to the body. If it takes the healthy heart ~0.5 second to entirely fill to a total of 120 ml blood during the diastolic phase, it will take another ~0.3 seconds to contract and eject – "systolic phase" – 70 ml of that blood to maintain a steady flow to the body. The heart never completely empties as residual volume is present at all times. In HF patients the FRANK-STARLING RESERVE is limited, which is detrimental to maintaining a steady blood perfusion to the body (cardiac output). Exploring deeper into the sarcomeres reveals the myofilament proteins. These come in two types: thin- (actin-containing) and thick- (myosin-containing). Molecular interactions between the actin and myosin proteins ("cross-bridges") generate the contraction of the sarcomere. Cross-bridges activation is regulated by Ca^{2+} and the MYOCARDIAL ENERGY RESERVE that regenerates ATP from ADP.

As it will be seen, it is the interplay of SARCOMERE LENGTH, Ca^{2+} and MYOCARDIAL ENERGETICS that regulate the diastolic function of the heart. When any, or several, of these mechanisms fail DIASTOLIC DYSFUNCTION follows.

[1]Stiffness - The rigidity of an object to which it resists deformation in response to an applied force. The SI unit is N/m.

[2]Stress or Pressure - The force exerted on a material normalized to the area over which the force is acting. The SI unit of stress is the pascal (Pa). 1 Pa = 1 N/m^2. Force applied perpendicular to the material surface – compressional or extension stress. Force exerted parallel to the surface - shear stress.

[3]Elasticity - The property of a material to deform in response to an applied force and returns to its original state when the force is removed.

Edited by Christopher N. Johnson

"Perfect for a novel or a biography after you have passed away, but way overboard for a grant [Rubicon] proposal!"

Coen Ottenheijm